

The promised land needs building

The peace agreement over Palestine has been widely taken as a sign that technical and financial assistance is needed urgently in Gaza; the Israelis have a bigger and special part to play.

Is Palestine (in the wider geographical sense) really a land of milk and honey? A decade after the state of Israel was founded in 1948, many Israelis believed their patch of it amply lived up to the promises of the Old Testament. They had already demonstrated that the desert could be made to bloom with citrus fruit, melons and the other appurtenances of a market garden economy. Despite the humiliations of the Suez fiasco in 1957, and the regional hostility reinforced by that dubious adventure, many Israelis then proclaimed that if only the rest of the Middle East would cooperate, they could use the simple techniques of irrigation to bring pastoral prosperity to the whole region. Of course, they reckoned without two crucial developments in the succeeding decades: the remainder of the Middle East has been made prosperous by the size and, even more important, the low extraction cost of its oil reserves, while the hostility of Israel's neighbours has been made implacable by the passage of time. But now the market gardeners and their technical successors may have their chance.

Everybody knows that the peace agreement signed in Washington last Monday is a huge gamble for both parties, the Israeli government and the Palestine Liberation Organization (PLO). Many of those who dissent from it will be tempted by the now-standard recipe of extremists: use guns and explosives to undermine the convictions of those who support a peace agreement. One counter to that primitive argument is a rapid improvement of the prosperity of those economically disadvantaged by the status quo, which in this case means the Palestinians of Gaza and the West Bank.

The European Communities (EC), to their credit, have already acknowledged the need by chipping in US\$700 millions. No doubt the United States will find itself contributing even more. Much will be learned about future relationships in the Middle East when the time comes to pass around the hat among Arab states and those of the Persian Gulf. But the contribution that Israel itself can make to the rebuilding of Gaza and the West Bank is more important than its money value, and should not be overlooked.

That is where Israel's market gardeners and their successors matter. The demonstrations of the benefits of simple irrigation techniques on offer several decades ago are not what the sophisticated Palestinians now most need. The technology of sound public administration is something else, but jobs for the people are an even more urgent need. The cohorts of young people brought up to believe that throwing

stones at policemen is a way of life will be more likely to stay off the streets if they have jobs to go to. Israel, already embarrassed by the flow of immigrants in the past few years from Russia and elsewhere in Stalin's broken empire, is itself worried by domestic unemployment. But the Israeli business community could play a crucial part in the development of the modern industrial enterprises that will be needed to enable Gaza to stand on its own feet after the initial charitable contributions have been spent. A readiness to assist in making Gaza prosperous will be an important test of Israel's commitment to the agreement, but would be of more than symbolic value; mutual trade leads to mutual prosperity and is also political cement.

Israelis have been talking for several months of one intriguing possibility of this kind: the development of tourism in the Middle East. Their starting point is the reflection that it has not hitherto been possible for people from elsewhere to visit the great historical sites of the Middle East in a rational fashion. As things are, for example, a person cannot walk from Jerusalem to Damascus (a journey of some historical importance) without breaking the journey halfway, returning to Cyprus or Cairo for another negotiation with the visa authorities. Such restrictions are a nonsense. Israeli enthusiasts for tourism are right to say that, if they could be swept away, the whole Middle East would benefit. Even in the short run, developments along these lines will bring benefits. But it must be a long time, longer even than the time now set aside for a negotiation of the status of Jerusalem, before tourism will solve Gaza's problems. Job creation there is the urgent need. □

Strategy for research

The governments of France and the United States have independently set out to develop a strategy for research.

THE US Federal Council for Science, Engineering and Technology (FCSET) is colloquially known as "Fixit", but has not conspicuously fixed anything during its long existence. It is instead more a talking shop of powerful agency heads who meet from time to time to agree to continue to disagree. Vice President Al Gore now wants to change that (see page 195), and not before time. By the scale of its support, the administration makes a decisive mark on the pattern of US research. Nobody's interest can be served when, as this year,



the annual budget is studded with expensive projects (the Superconducting Super Collider, the space station and the Idaho Fast Reactor) which the Congress promptly casts into limbo, at least temporarily. At the very least, the administration would be better able to get its way if it had formed a coherent view of what it wanted. It might even, that way, arrive at wiser goals.

The government of France, which is even newer, is similarly exercised (see page 196), but with characteristically Gallic differences. The Minister for Higher Education and Research, François Fillon, wants to make contracts with research agencies on the basis of an explicit set of priorities to be decided after a period of public consultation. He promises to enliven next spring with public debates along the lines of those arranged by the Socialist government more than a decade ago. That earlier exercise worked well, but largely because one man, Hubert Curien, was the minister in charge for most of the time and had the weight to carry through the strategy agreed. Does Fillon have the stomach for that?

Interestingly, the recent public consultation in Britain was less concerned with content than with procedure, but those who organized the exercise were surprised at the readiness of working scientists to offer opinions for the way in which research support should be organized. That is not at all surprising; do not researchers have a vivid interest in how the money that supports their work is spent? But research goals have yet to be divined, by the as yet untested mechanism of 'foresight' analysis. But in due course, Britain is also promised a research strategy.

What will happen if all three turn out to be the same? That, of course, is the extreme worst case. In reality, for example, neither France nor Britain is likely to decide to follow the United States in spending the best part of \$10 billion on a new particle accelerator. No formal papers on the topic would be needed to tell them that they cannot afford it, that it would be irrelevant to most other goals and that they are, in any case, committed to sharing in such an enterprise at CERN, the European High-Energy Physics Laboratory at Geneva. What that implies is what strategy-makers everywhere should acknowledge at the beginning of their task: that there is no point in designating as priorities research ambitions for which the facilities do not exist and for which there is no fund of enthusiasm within the research community.

Those are the tests by which the development of governments' strategies for research should be judged. On the face of things, the French device of public consultation about content seems the best, although there are some in Britain who would argue that 'foresight' (which is supposed to entail consultation among knowledgeable people) will have the same effect. At this stage, Gore's plan for the United States seems better attuned to the test of affordability than to an assessment of what the research community is enlivened by. Is he reckoning that the Congress, perhaps deprived of the chance to let big projects swing in the wind for a little while, will provide the research community with a platform for its own opinions? That would be no bad thing. □

BBC in trouble

Britain's best broadcaster may be making a mess of its strategy to win a new charter on favourable terms.

THE British Broadcasting Corporation (BBC) is going through a bad patch from which it may not escape undamaged. The BBC may be the best broadcasting organization in the world. Certainly it has the reputation of being at once an innovative source of public entertainment and enlightenment and an objective source of information. But the organization has now run into trouble of a kind probably periodically inseparable from its distinctive and even anomalous constitution, but on this occasion exacerbated by its own misjudgements. Especially at a time when there is a need for a wider understanding of the social and economic value of research, an erosion of the competence of the dominant broadcasting organization would be tragic.

The BBC is not a state-controlled organization. Its income of £1.5 billion odd is rather almost entirely derived from user fees payable by people who own television receivers, who must buy an annual licence. Other smaller chunks of income come from the sale of programmes to other broadcasting organizations and from the Foreign and Commonwealth Office, which makes an annual grant for radio broadcasts aimed overseas. Technically, the British government has no control over the BBC's broadcasting, which is the responsibility of its staff, themselves responsible to a board of governors, mostly lay-people of miscellaneous interests. But in reality, the government has three sanctions; it appoints the governors, it agrees (or disagrees) annually with the BBC's proposal of what the annual licence fee should be and, periodically, it has to rewrite the charter that allows the BBC to collect its chief source of revenue.

The benefits of this arrangement are that the BBC is in day-to-day control of what it broadcasts. The snags, as some see it, are the same; the BBC is not 'accountable' in any fashionable sense of the word. Politicians are especially offended that the BBC has an honourable record of showing up bluster for what it is. So it is not surprising that for the best part of a decade, there have been mutterings that the new charter, which must be agreed before the end of 1996, would be a truncated version of that now in effect.

Unfortunately the BBC's management seems bent on winning the battle for a continuation of the present arrangements by conceding the complaints. Able people have been turned prematurely into pensioners and there has been created a complicated internal market — the name for a device by which overhead costs are attributed in detail to individual broadcast programmes and whose effect is to impede creative work and demoralize those responsible for it. A couple of years from now, the BBC's managers will no doubt be telling those responsible for the new charter that they have taught the staff a humiliating lesson. The danger is that, in doing so, they may implicitly acknowledge that the BBC is no less (and no more) accountable than the British Army — to the government. □

Clinton to head 'one-stop shop' for science and technology policy

Washington. President Bill Clinton is to chair a new National Science and Technology Council which is being set up to oversee US science and technology policy. The goal of the administration is to exert stronger central control over the \$76 billion that the federal government spends each year on research and development.

Plans for the new body, which go further than any previous initiative to coordinate the research activities of separate and often competing government agencies, are included in the report of Vice President Al Gore's National Performance Review, published in Washington last week.

Like other elements in Gore's package — explicitly intended to "reinvent government" — the plans for the proposed council have still to face scrutiny in Congress. Many are likely to see them not only as a threat to the independence of existing agencies, but to the power of the congressional committees that oversee these bodies.

The vice president will be a member of the council, as will the heads of the main science-funding agencies, such as the National Aeronautics and Space Administration (NASA) and the National Science Foundation. The council will also include senior representatives — probably deputy secretaries — of relevant government departments, including energy, commerce, health and human services, defence and agriculture.

Details of the plan will be announced next week by the Office of Science and Technology Policy (OSTP). A draft version will also be circulated of an executive order establishing the new council, which will replace the existing Federal Coordinating Council for Science, Engineering and Technology (FCCSET).

The consultation process for such administrative changes would normally require around six weeks. But OSTP officials say they hope to complete it more rapidly, and to issue the final version of the order early in October.

The contents of the order will indicate just how much power the new council aspires to hold. Important issues still to be resolved include the exact composition of the council, and whether it will be able to issue directives that will be binding on government agencies.

The tough language of the Gore report implies that the council will indeed be given such powers. It complains that the existing FCCSET "can't compel agencies to participate in projects, nor can it tell agencies how to spend funds". And it adds: "At a time of declining federal resources, experts in busi-

ness, academia, and government recognize the need for one-stop shopping for science and technology policy."

OSTP sees the council as developing into the principal national body for science and technology policy, comparable to such

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John Gibbons: presidential science adviser and architect of the new council.

powerful existing bodies as the National Security Council and the National Economic Council.

The need for such a powerful coordinating body has been frequently expressed in the past, and the council has been warmly welcomed by the scientific community. Frank Press, President Jimmy Carter's science adviser and a former president of the National Academy of Sciences, says: "The fact that the President will chair the Council will send a clear message through the government that science is a priority area."

The National Performance Review also promised legislation to give the council the teeth it needs, in the review's words, to "direct science and technology policy more forcefully". Some of the required legislation has already been drafted and privately considered by the relevant congressional committees. The new council will also absorb the work of two existing but moribund bodies, the National Space Council and the National Critical Materials Council.

The proposals are the brainchild of John Gibbons, the presidential science adviser and OSTP director, who has already made public his desire to strengthen the FCCSET coordinating panels set up by his predecessor, D. Allan Bromley (see *Nature* 363, 291; 1993).

At present, six FCCSET panels serve as forums for coordination between different government agencies in key technology areas. But, says the National Performance Review, they cover only 16 per cent of the total amount that the US government spends on research and development, and they have no power to tell agencies what to do. And

Gore's report says that the panels as at present constituted "lack the teeth to set priorities, direct policy, and participate fully in the budget process".

The administration's proposal to develop such powers is bound to meet resistance both within agencies and in Congress, however. Supporters of NASA, the Department of Energy and the armed forces, for example, can all be expected to resist fiercely any infringement on their independence.

But officials suggest that this resistance could be muted by constituting the council as a 'policy board' which would not direct spending as such. Instead, it would make its influence felt through the administration's powerful Office of Management and Budget, a representative of which would sit on the council, and through the existing inter-agency coordinating process.

The idea of a coordinating science policy body chaired by a president or prime minister is not new, having been attempted in the United Kingdom and Japan, for example. But such panels have had a mixed track record in effectively controlling the activities of strong and independently minded government departments. **Colin Macilwain**

UK launches research foresight initiative

London. One of the cornerstones of the British government's new strategy for science and technology was put in place last week with the first meeting of its Technology Foresight Steering Group, a committee whose creation was promised in last May's white paper (policy document) on research.

The group, chaired by Bill Stewart, the government's chief scientific adviser, is made up of two academics, two government scientists, two research council heads and three industrial research directors.

Based on the experience of Japan and other countries, the group will be responsible for putting together a programme which will, according to the Cabinet Office, "build a consensus on those generic technologies where new developments are likely to yield the greatest economic and social benefits to the UK in the long term".

William Waldegrave, the cabinet minister responsible for science, last week described technology foresight as "encapsulating the theme of partnership which is central to the white paper". A series of regional seminars is being held throughout the country to publicize the programme, and the group is due to report early in 1995. □

France seeks five-year research agreements

Paris. François Fillon, France's minister for higher education and research, announced last week that the government intends to develop a new national science strategy. At its centre will be a system under which the ministry will enter into five-year contracts with all public research organizations, defining fixed objectives and the means to achieve them.

According to Fillon, the main lines of the new strategy will emerge from a "national consultation" on research priorities, which was also launched last week. This exercise — based on a similar exercise carried out by President François Mitterrand's first research minister, Jean-Pierre Chevènement, in the early 1980s — will probably culminate next spring in a series of regional meetings, a national colloquium and a debate in the National Assembly.

Fillon says that the new strategy will put greater emphasis on the social, economic and military applications of research; he has already said, for example, that aeronautics research will get more money. Another aim, he says, is to make research organizations such as the National Centre for Scientific Research (CNRS) more accountable to government.

"One important change is that we will not, for example, be able to provide CNRS

with everything that its researchers want", says Bernard Decomps, director general of the ministry of higher education and research. Decomps says the ministry intends to list research priorities in order to make spending decisions.

The French government is unlikely to use the five-year contract system to increase central control over science; indeed, it claims that its goal is to give research organizations greater autonomy.

But there is also uncertainty over whether the Ministry of Finance will let Fillon allocate five-year budgets, as these would be incompatible with the requirement that all public spending be authorized annually by parliament. "The contracts will need a clause 'on reserve of budget'", admits Decomps, although he points out that the ministry has honoured similar four-year contracts with the universities.

Fillon argues that, if the French parliament endorses his plans, this will strengthen his hand in negotiations with the finance ministry, which has already accepted pluriannual commitments in other areas — such as defence — which need long-term planning.

Without such assurances, the new contractual arrangement would become little more than a five-year rolling plan. Nonetheless,

science administrators such as Pierre Papon, a former director of the CNRS who is now president of the marine research agency IFREMER, claim that the move is in the right direction, particularly because it would help research organizations with their medium-term planning.

Observers also point out that the impact of a five-year contract would be reduced if the Ministry of Finance is not persuaded to give research organizations greater say in the way they spend their budgets. At present, because parliament approves not only the annual budget but also the proportion spent on investment, salaries, overheads and so on, organizations such as the CNRS cannot increase spending on one area by cutting it elsewhere.

Budgetary freedom could also be crucial if research organizations are to subcontract research to individual laboratories, as the government promised in its election manifesto earlier this year.

Such a move would allow researchers to choose how to reach, for example, an agreed level of activity and publication. At present, a laboratory must spend fixed proportions of its annual budget on recruiting post-graduates, buying equipment and so on, and cannot hold over unspent money to the following year.

Declan Butler

Berlin plans major cuts in student numbers

Berlin. The government of Berlin is to cut back drastically on the number of student places offered by the city's universities over the next ten years. The proposed reductions are part of a law passed last month which aims to improve the overall efficiency of the universities.

Under the new law, Berlin's Free University, which now has 60,000 students, will lose 10,000 places, and the Technical University will lose 4,500 out of 40,000 students. Each university will also be required to reduce the number of teaching positions by an equivalent amount. But the Humboldt University in East Berlin is, as a former East German university, being given different treatment, and will in fact grow.

The 115,000 student places now offered by Berlin's three universities represents eight per cent of Germany's student population. But the number of registered students in the city is far higher. German law makes it difficult to turn down applications from any students who have the school-leaving certificate, the *Abitur*, and as a result about 150,000 students crowd Berlin's classrooms.

Manfred Ehrhardt, science minister for the reunified city which has only 4.4 per cent of the country's population, says that it can no longer afford to carry an unfair share of responsibilities. During the Cold War,

Berlin's strategic position meant that it enjoyed special financial support from the federal government which covered about half of its operating costs. Now, like all other *Länder*, it is being required to take full financial responsibility for education.

Despite the planned cuts, Berlin will still offer a relatively high proportion — six per cent — of the country's student places. The universities will be making their own decisions about where the cuts will fall. The Free University is expected to make most of the reductions in humanities departments. But the science faculty, in which about one third of all students are registered, will not go untouched. The university expects to leave between 100 to 200 academic positions empty as these become vacant.

The new law will penalize students who spend too long at university. A surcharge of DM100 (US\$63) per semester will be made to students who continue their studies more than four semesters beyond the recommended course length, which averages eight semesters. This figure will rise to DM600 after 20 extra semesters.

At the same time as cutting back on student numbers, Berlin is falling into line with government policy and adding nearly 9,000 places within its more technically

oriented higher education establishments, the *Fachhochschulen*. And its newly established *Berufsakademie*, a college offering intensive vocational training, hopes to increase its student numbers from 170 to 600.

Although the expansion of German universities in the 1960s took place under the banner of "equality and excellence", Ehrhardt says there has been more of the first than the second. He also admits that he would have liked to take his reforms much further, expressing admiration for Saxony's education and research minister, Hans-Joachim Meyer, who introduced much more radical university reforms in July which push his east German *Land* towards an élite educational system (see *Nature* 364, 563; 1993).

As a step in this direction, Ehrhardt has given Berlin's universities the right to admit only students who demonstrate a relatively strong performance in the *Abitur* in particular subjects, rather than having to accept an average, across-the-board grade.

The universities are now voting on this change. But Ehrhardt says he recognizes that, given the strong socialist presence in the city government, it is unlikely to accept a system similar to Saxony, where each department can now stipulate precise entry requirements.

Allison Abbott

Gene therapy gathers speed in Germany

Freiburg, Germany. Overcoming fears of public opposition that now appear to have had little foundation, both federal and state authorities in Germany — and at least one pharmaceutical company — have begun pouring substantial amounts of money into research on the therapeutic use of genes.

At least eight groups are now engaged in gene therapy research. And, as a result of such activity, concern is subsiding that both German scientists and German companies could be left behind in this field by European competitors elsewhere in Europe.

At the beginning of this month, for example, the federal ministry of research and technology (BMFT) announced that it is prepared to spend up to DM60 million (US\$37 million) over the next six years to support trials of gene therapy techniques.

The goal of the programme is to support the development of genetic probes and techniques for transferring genes into the human body in ways that will allow the treatment of diseases such as cancer and AIDS.

The BMFT initiative has been launched at a time when, despite continued widespread public opposition to genetic engineering in general, there has been little specific criticism directed at research on the medical applications of such techniques.

Research in gene therapy is already being supported by Germany's main source of funding for university research, the Deutsches Forschungsgemeinschaft (DFG). In particular, the DFG has provided DM 6.6 million to support a three-year research programme at the University of Freiburg into the molecular and cellular basis of host defence mechanisms against tumours.

One participant in the project is Roland Mertelsmann, a haematologist who was last year given the first — and so far only — approval for gene therapy trials in Germany (see *Nature*, 360, 702; 1992). Mertelsmann chose to use electroporation rather than a viral vector to get the gene into cells because he feared public opposition to the use of viruses.

He plans to inject a vaccine containing a mixture of inactivated tumour cells and transgenic fibroblasts into which the gene for the immunostimulating cytokine

interleukin-2 (IL-2) has been inserted. The goal is to provoke an immunological response to melanoma and its metastases.

The first efforts, however, have been disappointing. The system worked well in mice, activating cytotoxic T cells that went on to select and kill cancer cells. But experiments so far with human cells have been less



Roland Mertelsmann

successful. As a result, no tests have yet been carried out with human patients (although Mertelsmann says that he expects to start vaccinating melanoma patients "within the next few months").

As an alternative strategy, Mertelsmann has now teamed up with the German pharmaceutical company Boehringer Ingelheim in a deal that allows him to use company's patented receptor-mediated endocytosis method to treat renal cell carcinoma.

This technique has been developed by Max Birnstiel and Ernst Wagner at the Institute for Molecular Pathology in Vienna, an organization set up in 1985 as a joint venture between Boehringer Ingelheim and Genentech of Cali-

fornia, but now wholly owned by Boehringer.

In addition to Mertelsmann, several other German teams are already planning to use the same transfection method. One, headed by Eva-Bettina Bröcker, director of the skin clinic in Würzburg, hopes to begin experimental treatment next year using IL-2 genes on patients with melanoma.

Two teams in Heidelberg — one at the Medical Polyclinic, the other at the German Cancer Research Centre — are also planning to start gene therapy tests to treat melanoma. Other groups in Germany are planning experiments on the potential use of gene therapy to treat leukaemia and haemophilia, as well as metabolic disturbances such as hypercholesterolaemia.

So far, Boehringer Ingelheim is the only company to have publicly acknowledged its interest in gene therapy research. In addition, the Boehringer Ingelheim Foundation — in a move which its directors claim was entirely independent of the company's interest in the field — has decided to offer half of its grants budget to the University of Mainz to enable the university to set up a group of young scientists working on gene therapy.

Other pharmaceutical companies, many of whom are working with research groups in other countries to avoid Germany's stringent genetic engineering laws, say they are keeping a close eye on developments.

Robert Unterhuber

Ireland seeks advice on research

Dublin. The Irish government is setting up an independent group to review its science and technology policy following complaints that it has not been taking adequate responsibility for research. But critics are worried that the report may be too narrowly based on the needs of industry.

The review group will be coordinated by the Office of Science and Technology (OST), part of the Department of Enterprise and Employment within the Ministry for Industry and Commerce. According to Michael Fahy of the OST, the group will consist of between ten and 15 individuals, selected from both industry and from the scientific and technical communities.

The group will be asked to provide its recommendations within six months, and the government expects to respond to the report with a white paper (policy document) of its own within a further three months.

Fahy says that the decision to set up a review group reflects the OST's concern that science and technology has no champion in government now that the major research funding agency, Eolas, has been absorbed into a new industrial support body

called Forbairt (see *Nature* 364, 662; 1993).

But a head of steam has been building up all year. Research support groups and academic groups have been putting the government under considerable pressure, complaining that the Ministry of Industry and Commerce, responsible for all government-supported non-health research funding, has shown little interest in maintaining a strong basic research base in Ireland.

The Irish Research Scientist's Association (IRSA) says it doubts whether a report originating within the Ministry of Industry and Commerce will provide a wide enough scope for a national science policy. A spokesman for the association, Michael Hopkins from Dublin City University, says that other government departments, such as health, education and agriculture, which are more active in research and have more general interests in science, should be involved.

The IRSA is also concerned that active scientists may not be appointed to the review group. The make-up of the group has not been announced, but Fahy says that the OST does not intend to include representatives of all interested parties.

Allison Abbott

European Parliament plans 'science summit'

Brussels. The European Parliament is planning a two-day meeting in Brussels next month, to discuss how science and technology can contribute to the new challenges facing Europe. The meeting, described as a "science summit", will be attended by leading scientists, government science policy officials, and directors of funding agencies, as well as by the chairpersons of the relevant Parliamentary committees. □

Insurance companies fight for rights to genetic data

London. Representatives of Europe's leading insurance companies are meeting in Paris this week to discuss how they can head off legislation aimed at restricting their access to genetic information about those seeking insurance cover.

The meeting is a response to growing concern that such information could be used to discriminate against individuals whose genome reveals a susceptibility to particular diseases, particularly if this information falls into the hands of employers.

Denmark has already debated legislation making it illegal for genetic information to be passed to insurance companies or employers. Parliamentary groups in several other European countries — including the United Kingdom — are also discussing whether to make similar moves.

Further action is taking place at the European level. An amendment is being proposed to a report being voted on later this month by the energy and technology committee of the European Parliament which urges legislation prohibiting doctors and medical authorities from disclosing genetic information about a patient.

Insurance companies have already expressed their opposition to any such moves. They argue that, at least in cases where genetic information is obtained as part of a medical assessment, it should be treated like any other medical information. Not revealing such information, they claim, could invalidate an insurance contract — and would also be unfair on those with a clean genetic bill of health.

At present, the information available through genetic tests is unlikely to make a significant difference to the approach already taken by insurance companies. The main concern is over the use that insurance companies are likely to be tempted to make of genetic information in the future, particularly because the more sophisticated this information becomes, the more directly it will be possible to assess the health prospects of individuals.

Officials of the Association of British Insurers (ABI) point out that their members have no

intention of requiring genetic tests on individuals seeking insurance "within the foreseeable future". At the same time, however, they acknowledge that circumstances could change as testing becomes more accurate in its predictions, and more widely available.

"We cannot say that we will never ask someone to take a genetic test" says John Wagstaff, secretary of the ABI's medical affairs committee. "We just do not know about the future."

Some argue that, even though this prospect remains a number of years away, discussions should already be taking place between the insurance industry, genetic experts and consumer interest organizations to try to reach agreement on how to regulate the use of genetic information.

"There is a window of opportunity open at the moment; but, given the different — and sometimes opposing — interests between the different groups involved, it is not going to stay open for very long," says Peter Harper, of the Institute of Medical Genetics at the University of Wales College of Medicine in Cardiff.

But the insurance industry is nervous of the external controls to which this approach could lead. At present it is insisting that self-regulation should be sufficient. With this in mind, the ABI, for example, is in the process of drawing up a code of practice for its members on the use of genetic information.

A similar approach is likely to emerge this week from a meeting of the members of the European Insurance Committee, a trade body based in Paris. Franz Josef Werle, the deputy secretary general of the committee, says that companies are likely to prepare a joint position paper setting out their opposition to restrictive legislation before the end of the year.

But the limits of self-regulation are also coming under increasing scrutiny. The need for mutually acceptable guidelines on how the insurance industry should proceed, for example, is expected to be emphasized by a working party of Nuffield Council on Bioethics which is due to publish a report on genetic screening within the next few months.

In the United States, an ethics subcommittee of the human genome project has proposed that the insurance industry should adopt a temporary moratorium on demanding access to the results of genetic tests until further discussion is held on how this access should be regulated.

So far, however, the industry has not warmed to this proposal. Nor have similar calls yet been made in Europe. But every announcement of a new genetic discovery is strengthening the case for government intervention to ensure that rights are protected on all sides.

David Dickson

US blows cool on the prospects of rejoining UNESCO

Washington. Officials in the Clinton administration have been dampening down hopes raised by the recommendation of a State Department task force that the United States should rejoin the United Nations Educational, Scientific and Cultural Organization (UNESCO) in 1995.

The task force, chaired by assistant secretary of state Douglas Bennet, opposed immediate re-entry. This had been sought by many scientific and teaching organizations in the hope that such a decision could be taken before UNESCO's general conference in Paris next month.

According to the Bennet committee, the \$65 million annual cost of such a step would make it necessary to divert funds from other programmes, which the administration is not willing to accept. But the task force did keep a window open by recommending re-entry in 1995, when the United States will in theory have finished paying off its arrears to the United Nations.

Even the prospects of this happening, however, are doubtful. Officials in Washington point out that spiralling UN expenditure, for example on peace-keeping missions in countries such as Bosnia and Somalia, makes it unlikely that the arrears will be paid off, while much scepticism about UNESCO's effectiveness remains. "A go-ahead from on high would be sufficient, but there is no sign yet that the administration regards re-entry as a priority," says one congressional official.

Both the United States and Britain withdrew from UNESCO in 1984, complaining of management failures and excessive politicization of its programmes. Optimists at UNESCO are still hoping that, in the light of reforms carried out since then by the new director general, Federico Mayor, Clinton will announce a date for re-entry in his first address to the general assembly of the United Nations on 27 September.

Their hopes are shared by the organization's supporters in Britain, where the Foreign Affairs Committee of the House of Commons recently issued a report urging the government to rejoin, and claiming that previous objections to the activities of UNESCO have been overcome. "The debate about UNESCO's policy, budgetary and management problems has been settled, broadly in the organization's favour", the report says.

But the general view is that President Clinton will still hold back. And if a newly elected administration committed to building bridges with the UN does not champion UNESCO now, officials in Washington say it is unlikely to find the money in two years time either.

Colin Macilwain

Polish science

A recent article (see *Nature*, **364**, 748; 1993) gave the incorrect figure for this year's Polish research budget. The correct figure is 9,000 billion zloty, or roughly \$500 million. Also, the state committee for scientific research has 12 (not 14) members elected by the scientific community; the chairman and secretary of the committee are appointed separately.

Japanese industry wakes up to computer networking

Tokyo. A small, privately funded research institute is pioneering the use of international data networks in the social sciences, and in particular has become the first Japanese social science research organization to subscribe to Internet, the international network of networks.

The institute is gathering support at a time when both Japanese government and industry are showing a sudden interest in computer-based communications. The main factor leading to this shift in emphasis appears to have been the aggressive approach of the Clinton administration to networking in the United States, making it possible, for example, to download electronic publications directly from the White House.

Such a level of accessibility astonishes most Japanese. US policy has galvanized officials at the Ministry of Posts and Telecommunications into frenzied discussions on how to go about networking Japan. Their fear is that, if they fail to come up with an appropriately positive response to the Clinton initiative, they will be criticized for holding back the country's economic development.

The National Institute for Research Advancement by the Centre for Global Communications (Glocom) was founded three years ago as an offshoot of a graduate school called the International University of Japan. Although it has only five full-time researchers, the institute has already shown that it has the potential to play a key role in Japan's struggle to come to terms with information revolution.

The Japanese scientific community in general has been very slow to adopt computer networking (see *Nature* 363, 575; 1993). In university social science departments in particular, network connections are still virtually unknown.

But the rapid increase in the global significance of this new technology has already been illustrated by an international survey of policy research organizations carried out by Glocom itself and released at the end of August.

That survey showed that 51 per cent of such organizations already use electronic mail, that almost a third are connected to Internet, and that the rate of connection is increasing exponentially over time.

Izumi Aizu, Glocom's managing director, says that the institute wants to become a gateway through which Japanese social scientists and policy researchers can make their opinions and papers known — at nominal cost — to the outside world.

In particular, he says, publishing such work electronically through Internet will encourage interaction between Japanese researchers and their counterparts in other

countries. Thus, rather than being presented with the finished product, the rest of the world should be able to get some idea of what projects Japan has in the pipeline, as well as the opportunity to contribute to them.

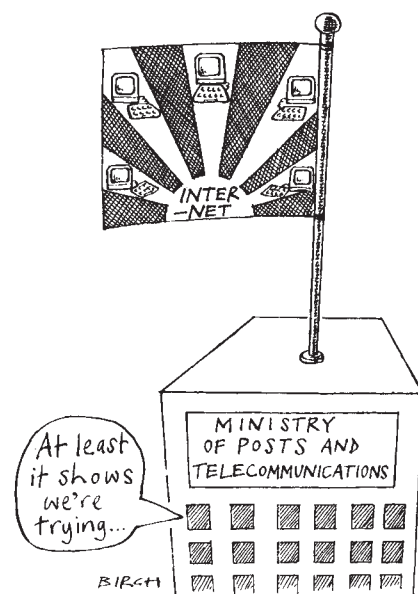
At the same time, Glocom has also embarked on a campaign to promote connection to Internet in Japan. At present, Aizu points out, Internet is not widely used. The main obstacle is expense: the leased lines required for connection to the network cost at least five times more in Japan than they do in the United States. Other barriers include restrictions on the type of institutions which are allowed to use the network, and the inevitable barrier caused by language differences.

Unusually for Japan, Glocom has decided not to apply for government aid. "We don't have time to do the paperwork," says Shumpei Kumon, the centre's acting director and a former professor of international relations at the University of Tokyo.

Kumon's previous position gave him first-hand experience of the red tape involved in education ministry grants. In contrast, Glocom's annual funding of approximately ¥100 million (\$950,000) comes almost entirely from private companies, including in particular Nippon Telegraph & Telephone (NTT), Tokyo Electric Power Co., and the department store group Saison.

The support received from these sources is in line with Kumon's prediction that while science has prospered from government support in the past, the role of government will in future diminish, and an increasing amount of funding for research will come from the private sector.

The companies that fund Glocom appear to have been motivated by an awareness that success in business depends increasingly on



how companies handle information. They are encouraged to participate in the centre's research rather than just donating money.

Kumon himself sees this in a broader perspective, and talks of establishing a new, all-encompassing branch of social science that will provide an intellectual framework for mapping the landscape of an increasingly information-oriented society.

Such ideas may sound grandiose. But Japanese businessmen and politicians seem to be listening. Last year, for example, the telecommunications company NTT turned down a proposal from Glocom for a joint project to study the potential of computer-based communications. This year, the company says it is more interested.

Bob Johnstone

■ William Waldegrave, Britain's cabinet minister responsible for science, is to pay a four-day visit to Japan at the end of September. Waldegrave plans to visit Tsukuba Science City, as well as a secondary school science class, and to hold discussions with leading scientists.

Grant winners get a surprise bonus

Munich. Scientists in the German state of Baden-Württemberg who receive support from the Deutsche Forschungsgemeinschaft (DFG), Germany's main source of funding for university research, are due for a pleasant surprise. The local state government has decided to introduce a bonus scheme under which all new DFG grant holders will have the money they receive topped up by a further five per cent.

Baden-Württemberg contains a number of major research centres including Heidelberg, Freiburg and Stuttgart. The bonus scheme is intended to preserve its status as a 'high-tech' state; research minis-

ter Klaus von Trotha hopes it will encourage university scientists to increase their efforts to seek external funding, rather than merely relying on that provided through the universities' own budget.

At present the scheme, which will be backdated to the beginning of this year, will run only up to the end of 1994. But von Trotha hopes it will be extended beyond this date, and also that it will be expanded to cover research grants obtained from other external sources. Last year universities in Baden-Württemberg attracted about DM550 million (\$344 million) of external funding.

Allison Abbott

China launches genome project

Beijing. The National Natural Science Foundation of China (NSFC), the country's main funding agency for basic research, has launched a national human genome project as part of its contribution to international efforts to sequence the human genome.

The decision to link up with sequencing efforts in other countries represents a determination by China's science policy-makers that the country should not be left behind in major frontier research fields. Zhou Guang-Zhao, president of the Chinese Academy of Sciences, has said that China's ambition is to win "gold medals" in what he describes as the "World Science Olympics".

China's participation in the human genome project could be significant. "The Chinese account for over one-fifth of the world's population, and the country has 56 ethnic groups," says one NSFC official. "As a result, no databank of human genetic information can be said to be complete without a detailed study of the Chinese human genome."

The project is likely to have important practical implications, as the Chinese are expected to contribute vital information that may lead to the early diagnosis and treatment of more than 5,000 hereditary diseases.

NSFC has not yet revealed how much money it is making available for genome

sequencing. However, it has already outlined a number of goals, including the collection and storage of the genomes of ten selected Chinese ethnic groups, and focusing on the genes that induce diseases peculiar to these groups.

Earlier this year, China announced that it was launching a programme to sequence the rice genome. The headquarters of the project is located in the life sciences research institute of the Shanghai branch of the Chinese Academy of Sciences.

Five satellite research centres have now been established in different parts of the country. Rice is China's staple food, and the Chinese rice genome project, described as second only to the world human genome programme, is intended to help produce improved rice varieties.

■ Jiang Ze-Min, state president of China and general-secretary of the Chinese Communist Party Central Committee, has pledged support for basic research in the country. Jiang, who is also president of the Chinese Central Military Commission and is a former minister for the electronics industry, made his remarks during a meeting on 12 August with the Nobel prize winners Gerd Binnig and Heinrich Rohrer, who were attending a conference on scanning-tunnelling microscopy held in the Chinese capital.

You Qin Li

Satellite disappearance remains a mystery

Beijing. A joint China-US enquiry into the disappearance of an Australian communication satellite, Optus B2, launched on a Chinese rocket in December, formally ended last month with a joint communique in which each side acknowledges the claim of the other that it was not to blame.

The \$138-million satellite was built by Hughes Space and Communications of the United States, but owned by an Australian company, Optus Communications. It was launched on 21 December last year by a Chinese Long March 2E rocket from the Xichang Satellite Launch centre in south-western China's Sichuan province. Approximately 48 seconds into the flight, there was an explosion on the spacecraft and the satellite disappeared from the monitor screen for reasons that have never been explained.

The latest joint communique suggests that the disappearance is likely to remain a mystery. It maintains that "the Chinese side finds no evidence that any flaw in the rocket has caused the failure and Hughes accepts this". But it also says that "Hughes finds no flaw in the manufacturing of the satellite has caused the failure and the Chinese side accepts this."

Both sides have agreed to conclude the enquiry, and to work together on launching another Optus satellite in the first half of

1994. To reduce the chances of similar incidents in future, Hughes and the China Great Wall Industrial Corporation (the Chinese contractor) have promised to make design improvements to the satellite and the rocket respectively.

The failure of Optus B2 seems to have had little effect on China's bidding to launch commercial satellites. The country has secured a significant share of the 66 satellite launches that are to be provided for the US Motorola Company between 1996 and 2002. It has also won contracts to launch three satellites for overseas companies in the next two years.

YQL

Nuclear accident law is passed

Beijing. China has formally enacted a nuclear accident law detailing the emergency measures to be carried out in case of an accident at a nuclear power plant. The decree, which was issued last month by Premier Li Peng, is the first of its kind in China, and is intended to provide guidance to the authorities on how to deal with possible nuclear accidents.

The law includes provisions covering the imposition of a state of emergency, the release of information and the operation of rescue work. It applies to any incident re-

Science gets the vote, but who was Einstein?

Beijing. A national survey carried out by the China Association of Science and Technology has shown that scientists and engineers enjoy high prestige among the public. Half of those surveyed said they would like their children to study science and engineering subjects at university, and subsequently to become scientists.

The survey was based on the results of interviews carried out over the past



Chinese scientists enjoy high prestige among the population.

year with 4,200 people between the ages of 16 to 69. Asked whether they considered that science and technology played an active role in achieving social progress, the majority of the interviewees said they agreed.

But such high expectations contrast with the low level of scientific understanding also revealed by the survey. Almost 20 per cent had no idea whether the Sun revolves around the Earth or vice versa. Fewer than half knew that it takes one year for the Earth to revolve around the Sun. And most had never heard of Albert Einstein.

On one point, the Chinese scored much higher than those in a comparable survey in the United States: 70 per cent said that they accepted Darwin's theory of evolution, compared with a US figure of 45 per cent. At the same time, however, nearly half of the Chinese believers thought that human beings existed at the time of dinosaurs.

YQL

sulting in the leak of radioactive materials into the atmosphere.

At present China has one nuclear plant in operation, the Qinshan nuclear power station, located just south of the country's largest industrial city, Shanghai. The country's second plant, in south China's Guangdong province, is expected to be completed next year. Its proximity to Hong Kong has given rise to public concern over safety which reached a climax during the 1989 student protest. But protests against nuclear power have since declined.

YQL

Spain calls attention to EMBL

SIR — The original concept that European molecular biology should attempt to emulate particle physics by trying to persuade governments to establish an international laboratory patterned on CERN (the European Laboratory for Particle Physics) crystallized in 1978 with the inauguration of the European Molecular Biology Laboratory (EMBL). Since then, EMBL has been renowned for high standards and scientific quality.

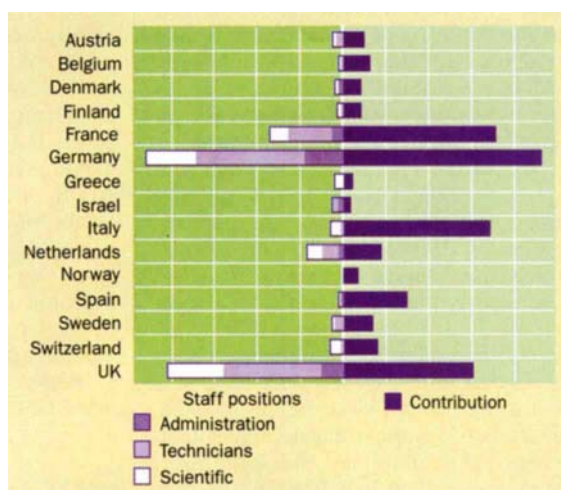
The rationale of such supranational projects relies on the assumption that either the development of science is too expensive for individual countries or that the whole is more than the sum of its parts and cannot be attained by individual countries. Although this probably holds for certain sciences, such as physics, it is not so clear that the same principle applies in biology. In fact, EMBL deals with the same things as national laboratories, approaching the same problems with the same tools, perhaps in grander surroundings.

The price that some of the member states must pay for the partnership is quite high. The budget of EMBL (see figure) is paid for by quotas based on the members' net national income (NNI). But three countries (Germany, United Kingdom and France) account for nearly 80 per cent of the benefits, as measured by the percentage of staff members. This illustrates the different attitudes of different member countries towards EMBL, as is clearly shown by Italy. Although it contributes 16 per cent of the EMBL budget, Italy had only five staff scientists in 1992. Spain's position is even worse — we contribute 7 per cent of the budget but have only one staff scientist.

This unequal distribution of benefits within EMBL was pointed out by the Italian delegation at recent meetings of the laboratory's governing council. And Spanish molecular biologists are asking whether it is reasonable to spend Spain's limited economic resources on an international laboratory without compensating advantages. This question is further stressed by the need for a country such as Spain, which is starting to make strides in science and technology, to have at home the best young scientists to boost scientific development. There are no simple answers, and clearly a substantial period (maybe 10 years) from our joining EMBL has to elapse before we can make a final evaluation.

This inequality needs attention, as the new EMBL director general seems to recognize. The problem now lies in the economic effort needed to implement his new initiatives, but the present economic recession makes things even more difficult.

We appreciate that science should be without borders and is international in its roots. Science requires international and multidisciplinary participation, but also the coexistence of differences. In that respect, science may even help to over-



EMBL staffing levels (left) compared to contributions for individual member states.

come the present crisis in Europe, by demonstrating the virtues of cohabitation.

International institutes must therefore be favoured. This can be achieved only if, like a living organism, EMBL evolves to ensure its existence in such a way that it will not be contested either by administrators or by scientists. That requires an actual system of quotas, which from our point of view, should be based on the level of participation and not on NNI, and also on the general principle that nothing that can be done by member states individually would be repeated at EMBL.

Eladio Montoya

Carlos Martínez-A.

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Problems at CNR

SIR — Crisis and chaos seem, unfortunately, to be endemic to Italian public institutions, and the Italian CNR (Consiglio Nazionale delle Ricerche) is no exception. We are writing to draw attention to the dismal plight of many postdoctoral research scientists within the CNR.

During the past four years, the average age of CNR researchers has increased and is now 45. This is because of a marked decline in the number of new research opportunities. Consequently, young career scientists, many trained within the CNR on CNR fellowships, are being forced to accept lower-level technician positions in order to pursue research and remain within the institution. There are some 170 scientists with doctoral degrees doing research at CNR facilities who have appointments at the technician level. Although their scientific productivity is comparable to that of CNR staff scientists or university researchers, they are paid much less and chances of promotion to positions commensurate with their qualifications, experience and productivity appear to be remote. This appalling situation is leading to growing frustration, apathy and resentment and threatens to exacerbate the debilitating atrophy that is crippling the CNR and the future of scientific research in Italy.

Surveys and letters in *Nature*¹⁻³ over the past decade have drawn attention to inequities within the CNR and the urgent need for reform. Yet little has been done to improve the prospects for researchers, particularly younger scientists. We hope that the recent political changes in Italy will lead to radical changes in Italian science and we call upon Italian scientists, especially those in positions of influence, to press for immediate action. Our present situation is intolerable.

Giovanni Agati

Franco Fusi

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1. Walgate, R. *Nature* **303**, 109 (1983).
2. Walgate, R. *Nature* **314**, 489 (1985).
3. Milne, R. G. *Nature* **361**, 191 (1993).

PiA grants

SIR — May I draw the attention of those among your readers interested in clinical immunology to the 1993 Primary Immunodeficiency Association (PiA) research grants?

The PiA is the patient support organization and registered charity for those affected by primary immune deficiencies. In 1993, the PiA will be funding research again for its second year, when £40,000 has been allocated for projects in clinical immunology.

Further details and application forms can be obtained from me. The closing date for applications is 1 December 1993.

Robin J. H. Fanshawe

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Italian drug problems clarified

SIR — I should like to make the following comments on the allegations about me in articles on the scandals in the Italian health sector (*Nature* 364, 562 & 663; 1993).

(1) My resignation as chief executive officer of Fidia dates back to February 1991 and was totally unrelated to any of the financial difficulties now being experienced by the company. The statement that I was "... fired last year following financial difficulties ..." is totally incorrect and without foundation. My resignation was the consequence of a disagreement with the major shareholder regarding the continuity of significant investment in scientific research. Anyone can easily confirm that, under my guidance, Fidia became one of the foremost Italian pharmaceutical enterprises dedicated to advanced research in the neurosciences (see *Nature* 361, 766; 1993) and was in excellent financial shape up to 1991.

(2) The joint venture with Georgetown University was undertaken in 1985 to increase the effectiveness of the company's research and development efforts, accepting the challenge of operating directly in the most exacting and competitive scientific environment in the world. The cost of the venture was proportionate to the company's financial resources and represented less than 10 per cent of Fidia's total research and development budget. It is sad to see that some very promising research projects are now being stopped just when they are about to yield significant results to substantiate important medical applications.

(3) In the present state of affairs, it is not up to me to defend the scientific position of gangliosides as remarkable pharmacological tools in nervous tissue repair. However, it is simply not true that "... approval was never sought in the US ..." Under my management, two of Fidia's ganglioside preparations were released by the US Food and Drug Administration as investigational new drugs for the treatment of disorders of the peripheral nervous system, spinal cord injury and stroke. Encouraging results in some of these areas have been reported¹.

(4) As regards the alleged association with Guillain-Barre syndrome, it has been pointed out that the coincidence is explainable in terms of a protopathic bias, and accurate epidemiological investigations in Italy have shown the inconsistency of the allegation^{2,3}. It remains a mystery why Fidia did not make effective use of the information available to defend its ganglioside preparations against often ill-informed and clearly tendentious attacks.

(5) A complete account of my personal involvement in the corruption scandal has been made to the investigating magis-

trates. The episode in question dates back to 1990 and refers to a formal application by Fidia to increase the price of a phospholipid preparation which had not been reviewed for over 15 years — in spite of an almost tenfold increase in production cost. Payments were repeatedly invited by the private secretary of the Minister of Health to "keep the application moving". My decision to pay was taken solely in the interests of the company and was in line with that taken by the Italian, as well as multinational, pharmaceutical companies, pressed to make funds available for the financing of political parties, as a condition for the review of otherwise perfectly legitimate applications. Ironically, a new price was never fixed for the phospholipid preparation in question.

This disgraceful situation, which has now come to light thanks to the relentless efforts of the Milan magistrates, played a role in my decision, in 1991, to found a new pharmaceutical company, Lifegroup, intended to operate exclusively in open-market conditions. Lifegroup now manufactures and sells drugs not reimbursed by the Italian Public Health Service, and is dedicating substantial resources to scientific research in the areas of neuroimmunology and dermatology. This is the direct consequence of my belief that in Italy only the restoration of free market rules, based on drug quality, free pricing and fair competition, will effectively combat the political conditioning of the market-place and remove from the entrepreneurial environment distortions that damage the whole economic order of the Italian health care sector and do not benefit the ill.

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1. Geisler, F. H., Dorsey, F. C. & Coleman, W. P. *N. Engl. J. Med.* 324, 1829–1838 (1991).

2. Paolino, E., Govoni, V., Tola, M. R., Casetta, I. & Granieri, E. *Neuroepidemiology* 10, 105–111 (1991).

3. Granieri, E. *et al. Neuroepidemiology* 10, 161–169 (1991).

Allison Abbott writes: I regret the error in the date of Dr Della Valle's resignation from Fidia.

Wrong ideas

SIR — V. Koliadin (*Nature* 364, 96; 1993) defends Peter Duesberg's repeated querying of the widely accepted hypothesis that HIV causes AIDS, saying: "If a work expresses an unorthodox view, its chance of being published is quite small. As the history of science demonstrates, most landmark works have been at first regarded as wrong and intolerable."

But do scientists accept wrong new

ideas more or less often than they reject ideas ultimately proved correct? An article in the *British Medical Journal* published the day after Koliadin's letter coincidentally addressed this issue. Bernard Dixon reported (307, 137; 1993) that of the recent analysis by Juan Campanario (*Social Studies of Science* 23, 342; 1993) of 300 Institute of Scientific Information (ISI) "citation classics", only 5.7 per cent of the authors explicitly mentioned difficulties in getting their findings published by peer-reviewed journals. Even allowing for considerable error in reporting and the vagaries of ISI's selection methods, it seems that "most landmark works" are readily considered by the scientific community before their ultimate acceptance, and are not simply regarded as "wrong or intolerable".

Of course Duesberg may end up as part of the 5.7 per cent. But as a working hypothesis, the idea that HIV causes AIDS does not provide a preventive course of action which, when adopted, works. Although it is technically correct to dwell on philosophical niceties, it is to be deplored if such a course inadvertently detracts from human well-being.

Jonathan Cowie
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Population growth

SIR — The central theme of your leading article on population growth¹ was "coercion". The question is: has the West exhausted all other options? Donor assistance for family planning amounts to about \$560 million annually. This accounts for 1 per cent of all development assistance². According to the United Nations Population Fund³, the governments of developing countries ("corrupt governments"?) contribute 75 per cent of the current total expenditure on family planning. Even if the affluent countries do not increase but merely reverse their pattern of assistance from military to development programmes (usually 10:1), the prospects for population growth would become much happier.

What we need most today is understanding rather than coercion and the "white man's burden" approach. More critical appraisal is needed of the available options.

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1. *Nature* 362, 379 (1993).
2. Population Reports, Population Information Programme 1–2 (Johns Hopkins University, Baltimore, 1991).
3. United Nations Population Fund. *Global Population Assistance Report 1982–1989*, 90 (New York, UNFPA, 1991).

Making models of muscle contraction

Models of even the simplest biological systems madden bench biologists by what seems naivety and pretension, but some of them deserve attention for their more general attributes.

THE industry for making models of biological processes may not be nearly as productive as present circumstances require. Part of the trouble is evidently the huge gap that still persists between models that are simple enough to be calculated with reasonable accuracy and the reality of the problems in biology on which they are expected to throw some light. The model-makers may spend endless time (and even other people's Cray time) solving problems only to be told by biologists that the models are either hopelessly oversimplified or, worse, are "unhelpful".

A little charity is called for. Sometimes, even the most rarefied models can provide a way of looking at a real problem that would not otherwise be available. Who, for example, would claim that the simple molecular orbital model of benzene is an accurate representation of the electrons in the molecule? And who would be without it?

The eager band of physicists busily adapting classical models in physics to problems in biology may need extra indulgence. One of the latest of their products is an adaptation to the case of muscle contraction of a model originally introduced by Kramers for explaining the exponential dependence of chemical reaction rates on free energy differences, the Arrhenius effect as chemists sometimes call it.

The crude model for this problem is that of a particle trapped in a potential well. Usually, the problem is one in classical mechanics. The question does not arise of whether the particle can tunnel through the potential barrier as an α -particle does (through the confining barrier of the strong nuclear force) in radioactivity, but is that of whether, and with what probability, it will acquire from its surroundings the thermal kinetic energy to escape. Over the years, this simple model has been found illuminating in a variety of unexpected fields; the motion of impurity atoms in solids is one example.

To repeat, what follows needs charitable reading. Marcelo O. Magnasco, who divides his time between the NEC research laboratory at Princeton and the Rockefeller University in New York, has devised an elaboration of the Kramers model to deal not just with muscle, but also with molecular motors generally, notably those by which the transport of chemicals is effected in eukaryotic cells (*Phys. Rev. Lett.* **71**, 1477; 6 September 1993). Magnasco's purpose is to plead the case for the unintuitive behaviour of objects such as molecules in the

"Brownian domain". He puts it vividly like this:

In the macroscopic world, energy need not be spent to support a force; any object resting on a table is an obvious example. But in the Brownian domain, a hypothetical microscopic object on a microscopic table will not rest but rather dance around until it reaches the end of the table and falls off. If we wish to confine the object to the table top, we are obliged to pick it up from the floor and place it again on the table, thereby spending some energy. This is of course not a permanent solution: the object will keep falling. In order to expend a force, energy needs to be spent constantly.

Magnasco's model is that of a ratchet on a molecular scale, best visualized in one dimension as a periodic potential energy in whose profile the repetitive units are asymmetrical; they might, for example, be 'V'-shaped, but with one arm making a different angle from the other to the vertical. Macroscopically, such a ratchet behaves unsymmetrically; less force is needed to pull an object over it in the direction of the arm with the smaller gradient than in the opposite direction. A generation of Victorian gadget-makers knew that.

What happens in the Brownian domain? Suppose that Kramers' model of a particle in a single box is replaced by that of a particle in a periodic box, each unit of which has the asymmetrical profile of a ratchet. The particle, being molecular, is also immersed in a thermal bath. Then strange things happen. For one thing, Newton's Second Law no longer applies in its simple form. The faster the particle is moving, the faster it will lose energy to its surroundings.

If one insists on describing only the motion of the particle that defines the state of the ratchet, and not that of the uncounted molecules in the surroundings with which it interacts, then the situation is more like that of the motion of a particle in a viscous liquid. In particular, the gradient of the potential energy determines the *velocity* rather than the *acceleration* of the particle. But only partly. Brownian exchange of energy between the particle and its surroundings must also be counted, which means a time-dependent force of necessity comparable with the forces generated by the ratchet and whose properties can be described only statistically as those of 'white noise', whose magnitude is necessarily an increasing function of the temperature.

There is a flourishing if minor industry in the handling of equations of the type

thrown up by these statements, which are known as Langevin equations. The interest of the present case is when the velocity of the particle representing the state of the ratchet is determined not only by the gradient of the potential energy and the white noise forces but also by a third force, which may at one extreme be a constant and, at the other, 'coloured' noise — white noise with some parts of the spectrum missing.

Marvellously, Magnasco is able explicitly to solve the simplest case of a ratchet (with a repetitive 'V'-shape with straight-line arms) and with a variety of third forces. He confirms the expectation that the ratchet begins to move before the temperature of the surrounding heat-bath responsible for the white noise reaches that equivalent to the height of the potential barrier in the ratchet. It is more interesting, and not intuitive, that there should be a temperature at which the movement of the ratchet in the general direction of the third forcing influence is a maximum. At too low a temperature, the chance that the ratchet will be lifted over the potential barrier by external noise is too small to matter. But if the temperature is too great, the ratchet goes into 'overdrive', and is more likely to slip backwards, even against the influence of the third force.

More complicated cases are dealt with by numerical simulation, largely with the objective of showing that the rate at which the ratchet on the average moves generally increases with the intensity of the forcing influence. The importance of that is to demonstrate that different forcing influences have similar effects. The general conclusion, on which purists may demand further proof, is that it is a sufficient condition for a molecular ratchet to move in the preferred direction, that the third force even if it can be described only statistically, should have longish time correlations built into it.

What does that say about the molecular motors in cells, muscle cells in particular? Magnasco himself says that the question "is still under investigation". Others may take a more optimistic view on two scores. For one thing, the general idea that there may be an optimum temperature for the operation of molecular ratchets will bring modest rejoicing. And some may even go looking for some system in a real cell in which the description of a real ratchet can be pinned down. They may even find substance in the guess that time-correlation follows from linkage with molecules of ATP followed by their hydrolysis.

John Maddox

Fishing for fresh catalysts

Thomas R. Cech

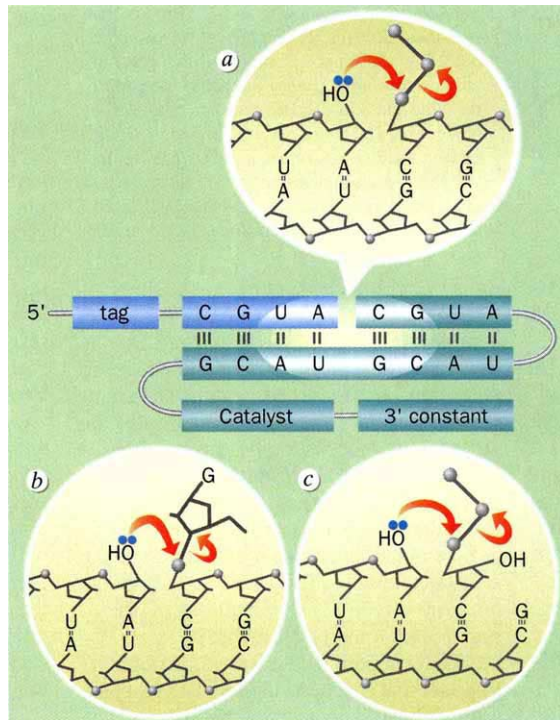
RNA enzymes can catalyse biochemical reactions with impressive efficiency, but how large is the catalytic repertoire of RNA? Evolution of RNA in the test tube provides an exciting new approach to finding the rare sequence that possesses a desired property. Now, in a paper in *Science*¹, Bartel and Szostak report the use of evolution *in vitro* to isolate a novel RNA ligase composed entirely of RNA.

In vitro evolution relies on the unique ability of RNA both to encode information and to fold into active conformations; that is, both genotype and phenotype reside in a single molecule. Experimental approaches¹⁻⁹ generally start with the construction of a large library of semi-random RNA sequences. The molecules are then challenged to perform some task, and the winners are collected (anthropomorphic language runs rampant in this field). The task requires the RNA to form a specific binding pocket and, in some cases, to accomplish a chemical transformation. The rare winners are converted to DNA, amplified by the polymerase chain reaction (PCR), and transcribed back into RNA so they can be subjected to additional rounds of selection. Each generation of molecules is further enriched in individuals that are fit to perform the task concerned. Generation times are short, as short as 1–3 days depending on the graduate student's perseverance, and populations are large, so within weeks the bulk population is readily performing a function that was perhaps originally undetectable.

This is an approach steeped in ignorance, and without apology: no understanding of RNA structure or catalytic mechanism is required to find a molecule that meets the preset criteria, just as one doesn't need to understand protein folding to raise an antibody that recognizes a specific antigen. The creativity comes in designing an experimental strategy that, when set in motion, will yield the desired result. The approach has a historical precedent in the elegant work of Sol Spiegelman, who used Q β replicase to evolve RNA with new replicative traits¹⁰. But with the availability of PCR, the basis of selection can be largely detached from the replication process (although of course the winning molecules must also be replicable).

In vitro evolution has allowed the isolation

of RNA molecules that form binding sites for small ligands, such as organic dyes or mononucleotides^{3,4}, or that are themselves ligands for polypeptides⁵. Single-stranded DNA selected by similar methods can rival RNA in simple ligand-binding tasks^{11,12}. However, we now know enough about higher-order RNA structure to appreciate that the 2'-OH



Template-dependent phosphodiester bond formation (a,b) catalysed by RNA (a, the Bartel–Szostak system; b, the group I ribozyme reaction, which is analogous to the reversal of the first step of RNA self-splicing) or (c) catalysed by contemporary protein polymerases and replicases. Dots represent phosphates, pentagons represent ribose sugars, and base sequence is arbitrary. In a, the catalyst region of the RNA is a collection of random 220-base sequences. Any sequence that can catalyse the reaction indicated becomes marked with the 5' tag, which is then used to purify it from the unsuccessful molecules. The 3' constant region and the 5' tag serve as binding sites for PCR primers, allowing amplification.

group is an important handle for building a complex RNA tertiary structure (see, for example, ref. 13), the sort of structure that may be needed for sophisticated catalytic activity.

In this even more challenging arena of transition state stabilization, the earliest successes were all based on improving or revising known RNA-catalysed reactions. Variants of the group I ribozymes have been selected to cleave single-stranded DNA with higher efficiency, to operate in an environment rich in Ca²⁺ instead of Mg²⁺, and to reactivate RNA-ligation activity in a ribozyme subjected to considerable size reduction⁶⁻⁸. Novel RNA

motifs have been selected to undergo site-specific cleavage by Pb²⁺ (ref. 9), a reaction that has a precedent in the Pb²⁺ cleavage of transfer RNA.

But how about reactions not known to be RNA-catalysed in nature? Bartel and Szostak¹ wanted to find a better ribozyme polymerase to further their goal of demonstrating RNA self-replication, a proposed early step in the origin of life. Although ribozymes derived from group I introns efficiently make 5'–3' phosphodiester bonds in a template-directed manner (see, for example, ref. 14), such

reactions suffer from an inherent limitation: the bonds being formed are no more stable than those being broken in the reaction. Although one can push such reactions forward by mass action, the thermodynamic driving force is far less than that at the disposal of modern-day protein polymerases, which consume nucleoside triphosphates to form phosphodiester bonds.

So Bartel and Szostak designed a system (a in the figure) to select for molecules that could catalyse the attack of a 3'-OH group on the α -phosphorus atom of a 5'-triphosphate. The task being demanded is not unlike that performed by ribozymes derived from group I introns^{8,14} (b in the figure), but the chemistry is that of contemporary RNA polymerases and replicases, with pyrophosphate as the leaving group (c in the figure). Successful ligation results in attachment of a specific oligonucleotide 'tag' to the catalytically competent molecule, which allows the winners to be collected on an affinity column decorated with single-stranded DNA complementary in nucleotide sequence to the tag. After ten generations of affinity selection, some of which included additional mutagenesis, the population was performing ligation with a half-life of a few minutes, whereas the uncatalysed reaction occurs with a half-life of 33 years — a million times improvement in performance.

In the course of the selection, the original pool of about 10¹⁵ different sequences had been winnowed predominantly to a single family of sequences. The structures of the successful ligators have yet to be determined; perhaps some will bear resemblance to group II introns, which have some ability to catalyse a similar reaction¹⁵.

What next? Now that we know that RNA can catalyse the reaction of a 3'-OH and a 5'-triphosphate, that it can bind a template-primer duplex and that it can also bind mononucleotides, it seems feasible that these properties could be combined to provide an RNA replicase com-

posed exclusively of RNA (*c* in the figure). Known nucleotide-binding sites in RNA are nucleotide-specific^{4,16}, so it remains to be seen how difficult it will be for RNA to build a site that binds the four nucleoside triphosphates with similar affinities.

Two fundamental cellular reactions that occur in ribonucleoprotein complexes have been proposed to be essentially RNA-catalysed: splicing of nuclear messenger RNA may be catalysed by small nuclear RNAs¹⁷, and peptide-bond

formation may be catalysed by the large ribosomal RNA¹⁸. Several groups are now using selection *in vitro* to try to recapitulate these reactions in RNA-only systems. These experiments have evolutionary implications, and will provide a better understanding of the range of chemical reactions amenable to RNA catalysis. □

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1. Bartel, D. P. & Szostak, J. W. *Science* **261**, 1411–1418 (1993).
2. Joyce, G. F. *Gene* **82**, 83–87 (1989).
3. Ellington, A. D. & Szostak, J. W. *Nature* **346**, 818–822 (1990).
4. Sassanfar, M. & Szostak, J. W. *Nature* **364**, 550–553 (1993).
5. Tuerk, C. & Gold, L. *Science* **249**, 505–510 (1990).
6. Beaudry, A. A. & Joyce, G. F. *Science* **257**, 635–641 (1992).
7. Lehman, N. & Joyce, G. F. *Nature* **361**, 182–185 (1993).
8. Green, R. & Szostak, J. W. *Science* **258**, 1910–1915 (1992).
9. Pan, T. & Uhlenbeck, O. C. *Nature* **358**, 560–563 (1992).
10. Mills, D. R., Peterson, R. L. & Spiegelman, S. *Proc. natn. Acad. Sci. U.S.A.* **58**, 217–224 (1967).

11. Bock, L. C., Griffin, L. C., Latham, J. A., Verma, E. H. & Toole, J. J. *Nature* **355**, 564–566 (1992).
12. Ellington, A. D. & Szostak, J. W. *Nature* **355**, 850–852 (1992).
13. Pyle, A. M., Murphy, F. L. & Cech, T. R. *Nature* **358**, 123–128 (1992).
14. Doudna, J. A. & Szostak, J. W. *Nature* **339**, 519–522 (1992).
15. Morl, M., Niemer, I. & Schmelzer, C. *Cell* **70**, 803–810 (1992).
16. Bass, B. L. & Cech, T. R. *Nature* **308**, 820–826 (1984).
17. Madhani, H. D. & Guthrie, C. *Cell* **71**, 803–817 (1992).
18. Noller, H. F., Hoffarth, V. & Zimmnick, L. *Science* **256**, 1416–1419 (1992).

POLYMER SCIENCE

Travelling by tube

Julia Higgins and Tom McLeish

ON page 235 of this issue Russell and co-workers¹ address a question that has been entertaining polymer scientists since the 1970s. It was Pierre-Gilles de Gennes², Sam Edwards and Masai Doi³ who introduced and developed the concept of 'reptation' — that a polymer molecule in the molten state is effectively confined in a tube (Fig. 1), and that the material flows by snake-like motion of the molecules out of their tubes. Is this true in practice?

Russell and colleagues look at the interdiffusion of two juxtaposed samples of molten polymer. If the molecules really move in this way, then the end-portions of the chains will cross the interface markedly before the middles do. They use an ingenious labelling with deuterium to distinguish the ends in one sample and the

middle in the other, providing differently labelled molecules on either side of the interface. The deuterium profile across the interface is observed by dynamic secondary ion mass spectroscopy, which works by digging through the interface with a beam of oxygen ions and detecting the flux of emitted deuterium ions. The deduced deuterium profile is indeed consistent with reptation.

Using selective labelling of portions of a polymer chain at an interface is new, but the idea of distinguishing between ends and middles by labelling with deuterium has been used in the bulk to detect differences in rates of changes in molecular orientation between central and end segments⁴. Here, the method was to look at both the scattered and absorbed parts of an infrared beam passed through the polymer melt, and again it was found that the chain ends reoriented faster than the centres.

Such experimental data are persuasive, but do require careful analysis. For example, the qualitative features of both experiments would still hold if the tubes were not there: the connectivity of a polymer chain ensures that for motions within the overall dimensions of a polymer coil, chain ends move faster on average than middles. The existence of tube con-

straints both increases the amplitude of the resultant deuterium profile and slows down the rate at which it emerges. To remove some of these uncertainties we will need experiments comparing molecules of different molecular masses which are not entangled, together with quantitative comparison to calculations

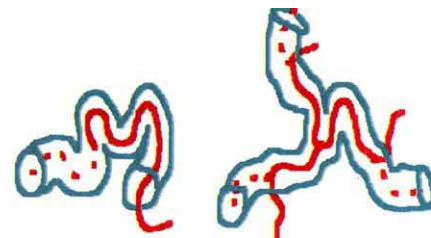


FIG. 2 The characteristic dynamic processes for entangled polymer motion within a tube. On the left a linear molecule escapes by reptation, on the right a branched molecule escapes by arm fluctuation.

in the presence and absence of tube constraints.

Most evidence for the tube model is indirect and comes from bulk properties, particularly rheological properties, of polymer melts. The model has been spectacularly successful, not least because it is easily visualized — wriggling snakes or bowls of spaghetti are images accessible to the scientist, the engineer and the lay person alike. As a consequence, tube theory has generated intense discussion and given rise to many ingenious experiments, such as those of Russell and colleagues.

Branched polymers provide a particularly sensitive rheological test-bed. Branched topologies of long-chain polymers must also 'feel' the tube constraint, but the molecules cannot reptate because their branch-points effectively pin them down. The simplest example is the star polymer (Fig. 2). Such polymers are a sensitive test of the existence of the tubes. Their dominant motion is a 'breathing' of the free ends in and out of the tube. The very slow timescales of the breathing modes produce the high viscosities of entangled stars, and can be quantitatively explained by the tube model⁵. Linear polymers too have seen successes. One challenge was to explain the exact form of the relationship between viscosity (η) and molecular weight (M), $\eta \propto M^{3.4}$, through the tube model, and recently the combination of breathing modes and reptation⁶ has come up with the correct effective power law.

Two other, more direct methods have been used to demonstrate the existence of the tube: experimental neutron-scattering and computer simulation. Hydrogen and deuterium scatter neutrons differently, so partial labelling with deuterium (as in Russell and colleagues' experiment) gives a way of observing one molecule sur-

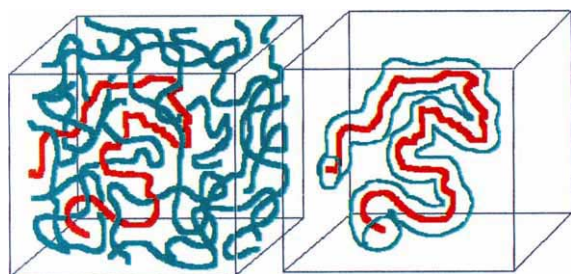


FIG. 1 The tube model supposes that any given polymer chain (such as the one labelled red here) when constrained topologically by its neighbours (labelled blue) behaves as if it were temporarily trapped in a tube that follows its own contour. The many-body system on the left is replaced by the single-body system on the right.

rounded by many others. To observe dynamics, the neutron spin-echo technique must be used, and experiments some time ago⁷ on two polymer mixtures tended to support the idea of the tube. In one, the surrounding chains were short, and ordinary brownian fluctuating motion was observed. In the other, the surrounding molecules were long enough to be thoroughly entangled both with the labelled molecules and with each other and thus to form an effective tube. In this case motions involving excursions longer than about 3 nm were severely hindered. This length scale corresponds closely to the dimensions of the tube required to fit the viscoelastic data for the same polymer. Further measurements on similar samples using an upgraded spectrometer have revealed further details of the constraints⁸. The timescale of the spectrometer is limited to motion faster than about 10^{-8} seconds, however, so the slower reptation of the molecules out of their tubes is inaccessible. It is just these slower motions that are observed in Russell and collaborators' work by comparing a series of instantaneous 'snapshots'. There is no limitation on the time interval between them.

Molecular dynamics simulations, in which equations of motion are written for each segment of each polymer chain, can be successfully applied to dense polymer melts, but they require enormous computing power, and are still limited to times short compared with motion out of the tube. Recent results⁹ do show that at short times, central parts of molecules are confined to a bundle of paths whose diameter is that of the expected tube.

The idea of tubes in polymer melts seems to be here to stay, but experimental and theoretical challenges remain. The large polydispersity in molecular weight of most industrial polymers makes more pressing the need to understand the dynamics of the tube itself as shorter, faster chains release the tube constraints on longer, slower ones. Equally, some inroads have already been made into the nonlinear properties of polymeric fluids using tube theory. A very recent example is a class of surfactant solutions in water in which the surfactant molecules aggregate

into long cylinders which then self-entangle, imparting a high viscosity to the solution (this is the basis of some sham-poops with high water-content). The effective tubes in such fluids give rise to unusual elastic effects under conditions of high shear¹⁰.

Experimentally we still await direct molecular observation of tubelike motion,

but despite some residual questions experiments seem to be getting very near to observing reptation itself. Perhaps polymers really do move like snakes. □

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EVOLUTIONARY GENETICS

Drunken walk of the diploid

Laurence D. Hurst

DIPLOIDS typically wend their way towards haploidy in a manner reminiscent of drunkards returning from an evening's revelry: one step backwards, two steps forwards. The first step in classical meiosis is to duplicate the chromosomes to produce a tetraploid cell. Two reduction divisions later four haploid progeny are produced. Some organisms such as red algae and microsporidians do not perform a classical meiosis, but these too avoid the most obvious route of splitting in two.

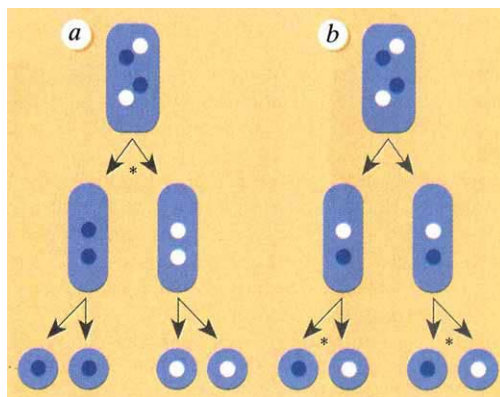
Why so? In two papers in the *Journal of Theoretical Biology*^{1,2}, David Haig proposes an attractive solution to this problem. The circuitry of meiosis is, he argues, necessary to prevent the invasion of a particular class of selfish gene.

Consider the two cells that are the product of the same meiotic division (that is, two meiotic sister cells). Consider also a gene that made its cell inject toxin into her meiotic sister cell. This sort of selfish gene has been termed a SisterKiller², and

the crux of Haig's argument for why meioses are not a simple, single step is that such a division is very vulnerable to SisterKillers.

In a diploid cell heterozygous for any given gene, the division of one-step meiosis will always be reductional; that is, if you can specify which one of the two alleles at a locus is in one of the haploid progeny, you can be sure that the other cell must contain the other allele. In the alternative (equational) form of division, the two progeny cells do not differ at the heterozygous locus under consideration. That one-step meiosis is reductional for all loci makes life easy for SisterKillers, for a mutant gene that is just coming into a population and which destroys its sister cell will always be destroying a cell that does not contain a copy of itself. This SisterKiller could spread in the population even though it reduces the host's fertility.

Contrast this with classical four-strand meiosis involving crossing over. In regions away from the centromere, if there is crossing over between the gene and the centromere, then, for any given heterozygous locus, sometimes the first division will be reductional and at others it will be equational. If meiosis I was reductional, then meiosis II will be equational and vice versa. These divisions are thus referred to as being semi-reductional. That is, sometimes if



The advantage of increasing ploidy prior to random reduction divisions. Consider that the diploid individual is heterozygous for a given gene (*Ss*). Those haploid nuclei with *S* are indicated by blue circles and those with *s* by white circles. Imagine that there was only one round of endomitosis and so there are two copies both of gene *S* and of *s*. If the four sets are mixed and then the cell divides, one-third of the time the first division will be reductional (*a*), otherwise the second division will be reductional (*b*). A SisterKiller acting at the first division would kill its identical copy two-thirds of the time, but one acting at the second division will kill the copy one-third of the time. The more endomitoses before division, the lower the probability that any given division will be reductional and the lower the vulnerability to SisterKillers. It will, however, always be the case that the last division is the one most likely to be reductional. The probability that sister nuclei will contain different alleles after the final division is $2^n/(2^{n+1}-1)$ where n is the number of rounds of duplication from the original diploid set. If $n=0$ (one-step meiosis) then this probability becomes 1, if $n=1$, as above, it is 2/3 and so on. As the number of rounds of endomitosis go up, the probability that the last division will be reductional tends towards a half. An asterisk indicates a reduction division with respect to the *S* locus.

1. Russell, T. P. *et al.* *Nature* **365**, 235–237 (1993).
2. de Gennes, P.-G. *J. chem. Phys.* **55**, 572–579 (1971).
3. Doi, M. & Edwards, S. F. E. *J. chem. Soc. Faraday Trans. 2*, **74**, 1789–1801; 1802–1817; 1818–1832 (1978).
4. Ylitalo, C. M., Fuller, G. G., Abetz, V., Stadler, R. & Pearson, D. S. *Rheol. Acta* **29**, 543–555 (1990).
5. Ball, R. C. & McLeish, T. C. B. *Macromolecules* **22**, 1911 (1989).
6. O'Connor, N. P. T. & Ball, R. C. *Macromolecules* **25**, 5677–5682 (1992).
7. Higgins, J. S. & Routs, J. E. *J. chem. Soc. Faraday Trans. 2*, **81**, 757–767 (1985).
8. Richter, D. *et al.* *Phys. Rev. Lett.* **64**, 1389–1392 (1990).
9. Kremer, K. & Grest, G. *J. chem. Phys.* **95**, 5057–5086 (1991).
10. Spenley, N., Cates, M. C. & McLeish, T. C. B. *Phys. Rev. Lett.* **71**, 939 (1993).

a gene acts to kill the sister cell from meiosis I it will be destroying its identical twin. At other times, killing at meiosis I will, as with one-step division, result in copies of that gene only being produced by the meiosis. The same is true for meiosis II.

So four-strand division with crossing over makes life hard for SisterKillers. Confusion, it is argued, is the name of the game. Keep a gene guessing which product of meiosis it and its identical copy will end up in, and you can prevent that gene from taking action to kill the cell that it will not end up in. Many diverse forms of classical meiosis are understandable from this viewpoint², from the bizarre dance of the X chromosome in crane flies to the curious jiggling of chromosomes at achiasmate meiosis in *Drosophila* males.

The paradox of the diploid's drunken walk therefore has a solution (see also ref. 3). But not everything indulges in classical meiosis. How do these other organisms fend off SisterKillers? One technique¹, employed by some red algae, is to go through series of endomitoses (mitosis without cell division) which take the cell to a high ploidy before an extensive series of reduction divisions. The advantage of reducing down from high ploidy is that it will always be uncertain just which division is the real reductional one for any given gene (see figure). This uncertainty guarantees that a SisterKiller could be destroying a cell containing a clonal copy of the same SisterKiller gene, and the gene will gain no advantage over its wild-type competitor.

Microsporidians (unicellular protists) seem to use a similar but not identical technique. Two haploid cells fuse to produce a diploid. Assume that the diploid is an *Aa* heterozygote, which then goes through a mitosis but without cell division. There are now two nuclei, both of which are heterozygous. Within each separate nucleus the chromosomes then pair up with their homologue. The two nuclei fuse, and the paired-up chromosome combinations align to undergo two divisions to produce haploid progeny⁴. At the first division, the two copies of *A* could both go to the same pole or to different poles. If they go to the same pole, the second division will be the one vulnerable to SisterKillers; if to different poles, the first division will be vulnerable. However, because there is no information as to which way the division is going to go, SisterKillers will destroy their identical copy as often as they destroy their non-clone mate.

There is at least one big problem with all this theory — no one has yet found a SisterKiller. That, though, is not totally unexpected. As SisterKillers spread, the frequency of homozygotes for SisterKiller goes up, and in such homozygotes SisterKillers may be trying to annihilate each

other. Selection could then favour those that are immune to the toxin. All of the well-described, classical, meiotic drive genes (for instance Segregation distorter, *SD*, of fruit flies) are of this nature: one locus produces toxin and a closely linked gene provides the antidote⁵. Hence, SisterKillers might be selected to become more like *SD*. If so, we might expect these classical meiotic drive genes to be linked to the centromere, as it is the one part of a chromosome that always has reductional divisions at meiosis I and hence is vulnerable to SisterKillers. Perhaps it is significant that all of the well-described meiotic drive genes (bar one⁶) are linked to the centromere⁵. However, this point should not be overplayed because conventional theory of two-locus drive requires that the toxin and antitoxin loci are tightly linked⁷. A chromosome region with a recombination block, such as the centromere, is thus more likely to harbour classical meiotic drive genes than a region with free recombination.

In all, it seems reasonable that meioses of many different types have devices to arrest SisterKillers. The great strength of this explanation is that it is parsimonious, and makes rhyme and reason from apparent nonsensical diversity. Testing it would be tough, but one possible tack is to enquire into the genetics of heavily inbred organisms⁸. Under these circumstances SisterKillers will nearly always be

metaphorically shooting themselves (or at least their clonal copies) in the foot. Hence protection from SisterKillers will not be necessary and heavily inbred organisms could have developed different genetic systems⁸.

As Haig predicts that one-step meiosis is typically vulnerable to SisterKillers, if it does exist it might be in heavily inbred organisms. Haig argues¹ that, although there are claims that one-step meiosis is present in a variety of poorly understood unicellular organisms, there is no unambiguous report of it. Perhaps significantly, however, according to the original description of *Urinymphe*⁹, a protist with what might be a one-step division, this organism only ever selfs. Re-examination of this and other poorly understood meioses could well be fruitful. □

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1. Haig, D. J. *theor. Biol.* **163**, 15–31 (1993).
2. Haig, D. & Grafen, A. J. *theor. Biol.* **153**, 531–558 (1991).
3. Bernstein, H., Hopf, F. A. & Michod, R. E. in *The Evolution of Sex* (eds Michod, R. E. & Levin, B. R.) 139–160 (Sinauer, Sunderland, Massachusetts, 1988).
4. Canning, E. U. *BioSystems* **21**, 333–340 (1988).
5. Lytle, T. W. A. *Rev. Genet.* **25**, 511–557 (1991).
6. Agulnik, S. I., Agulnik, A. I. & Ruvinsky, A. O. *Genet. Res.* **55**, 97–100 (1990).
7. Charlesworth, B. & Hartl, D. L. *Genetics* **89**, 171–192 (1978).
8. Hurst, L. D. & Hamilton, W. D. *Proc. R. Soc. B* **247**, 189–194 (1992).
9. Cleveland, L. R. J. *Morphol.* **88**, 385–440 (1951).

NUCLEOTIDE REPEATS

Slippery DNA and diseases

Thomas A. Kunkel

FEW observations have had more impact on our view of the integrity of genetic information than those of the dramatic instability of repetitive DNA sequences in human diseases. Expansion and contraction of these 'microsatellites' is associated with cancer and several heritable diseases. The report by Strand *et al.* on page 274 of this issue¹, which comes from the laboratories of Petes and Liskay, provides one possible explanation. It suggests that the loss or gain of repeated DNA sequences occurs because errors resulting from strand slippage during DNA replication remain uncorrected as a result of defective post-replication heteroduplex repair.

The human genome contains many sequences in which one- to six-nucleotide motifs are tandemly repeated numerous times². Mono-, di- and trinucleotide repeats are unstable in colon cancer cells, and a gene on chromosome 2 has been implicated in this instability³. Expansion of trinucleotide repeats from a few to hundreds or even thousands of copies is associated with a number of heritable

genetic diseases⁴, including fragile X syndrome, Kennedy's disease, myotonic dystrophy, Huntington's disease and spinocerebellar ataxia type 1.

There is a good chance that repetitive elements will misbehave during DNA transactions. Microsatellite instability could involve unequal crossing over between repeats or DNA strand misalignment during replication or repair⁵. Here mutation rates could be affected by the probability of misalignment (step 1 in the figure); the probability of continued polymerization versus reversal by realignment or exonucleolytic proofreading and resynthesis (step 2); or the efficiency of heteroduplex repair (step 3). This third factor also operates on heteroduplexes generated during recombination. The contribution of steps 1, 2 and 3 to stability in prokaryotes for substitution mutations is about 100,000-, 100- and 1,000-fold, respectively.

Given that information on these processes with repeated sequences in higher organisms is sparse, the study by Strand *et al.*¹ is both timely and important. It

employs yeast, a powerful eukaryotic genetic system in which many DNA transactions are the same as in humans. The key result is that the stability of a repeated dinucleotide sequence is much reduced in mutant strains defective in heteroduplex repair. So one route to microsatellite instability is reduction in heteroduplex repair efficiency so that replication errors remain uncorrected. This is one possible cause of the 'genome-wide' instability

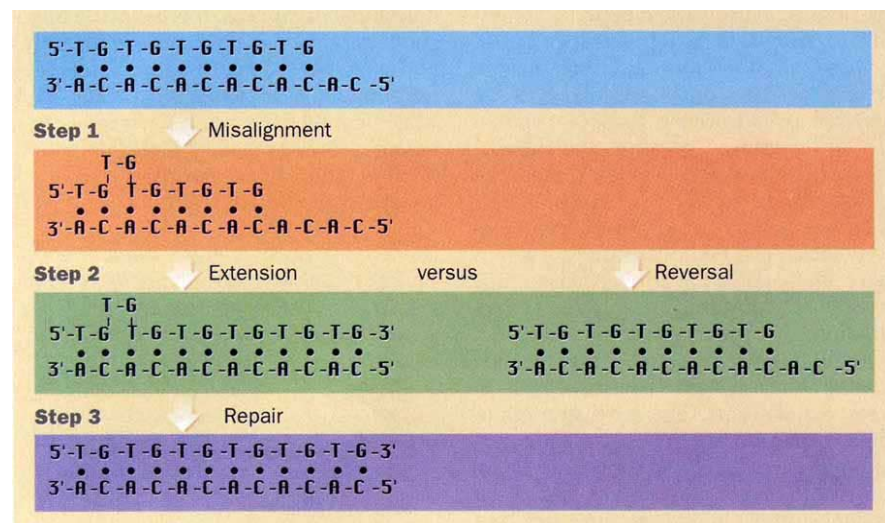
which step, if inactivated, might have the greatest effect on stability. Note that a model invoking misalignment predicts that discrimination by steps 1 and 2 should diminish as the number of copies of a repeat motif increases. This is because the misaligned intermediate might be more stable and proofread less efficiently. To the extent that heteroduplex repair is independent of the number of copies of the repeat, it could be increasingly impor-

issues. One wonders whether specialized proteins might exist to deal with DNA transactions involving the large amount of repeated DNA in higher eukaryotes. There are several distinct mismatch repair pathways in bacteria, yeast and humans⁷. Is there a system in humans to correct heteroduplexes in repeated elements and/or heteroduplexes containing a large number of unpaired nucleotides? Some of the diseases mentioned above are characterized more by expansion than contraction, yet there is evidence that replication misalignments within homopolymeric sequences lead to more deletions than additions⁸. What determines the balance between additions and deletions in microsatellites? Strand *et al.* begin to address this question by defining mutation specificity in one repeat, but more work is clearly warranted. For example, although we know that DNA can exist in many different conformations⁹, we have a limited understanding of the structures that might occur within repeats and how these might affect replication, heteroduplex repair and recombination.

Finally, cancer is a multistage disease requiring several mutations, and the hypothesis has been offered that cancer cells may have a mutator phenotype¹⁰. This possibility had little experimental support until microsatellite instability was observed in colon cancer cells. Does this mean that the hypothesis is correct but limited to certain mutations and/or locations in the genome? Are cancer cells also unstable for point mutations? If not, this would argue against a general defect in heteroduplex repair unless there are different controls on point mutations and microsatellites.

The attention of students from a variety of disciplines is now riveted on slippery DNA. Because more effort has been spent on substitution than misalignment mutagenesis over the years, we have some good ideas but few hard facts on mechanisms of microsatellite instability. In short, a number of possibilities remain open. □

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Three steps in discrimination against errors in slippery DNA. At the top is a template (lower strand) containing five copies of the dinucleotide (dA-dC). During 5'→3' synthesis of the complementary (dT-dG) strand (from left to right), strand slippage can form an intermediate containing unpaired repeat units stabilized by downstream base pairs. In the example shown, the unpaired nucleotides are in the newly synthesized strand which, when subsequently replicated, results in template expansion of the repeat sequence. Unpaired nucleotides in the other strand would lead to deletions. Dots represent hydrogen bonds.

associated with microsatellites in colon cancer. Defective repair could also contribute to instability in triplet repeats, although other mechanisms can be envisaged⁴ for the enormous expansion observed in some diseases.

The observations of Strand *et al.* do not preclude explanations for microsatellite instability other than defective repair. Far from it. As the authors note, the modest effect on repeat stability observed for a defect in exonucleolytic proofreading may reflect the special challenge of accurately replicating repeated elements. Misaligned nucleotides can be protected from the exonuclease by the terminal base pairs possible at repetitive sequences (see step 1). Thus, the contribution of proofreading to genome stability may be less for microsatellites than for substitutions. Using the analogy with *Escherichia coli* and implicit in the present study, most discrimination against microsatellite instability may occur at the polymerization step itself, presenting the greatest opportunity for instability through modification of the gene products that normally prevent misalignments from forming or being used as substrates for replication.

The present study leads one to wonder

tant, relative to the earlier steps, for stabilizing longer repeats. The effects observed by Strand *et al.* also call to mind that an *E. coli* mutator defective in proofreading generated so many heteroduplexes that heteroduplex repair became exhausted, yielding an extreme mutator effect⁶. This was seen when the cells rapidly proliferated but not when they grew slowly. Are there circumstances in eukaryotes when rapid proliferation could saturate post-replication repair capacity to yield a mutator phenotype?

So the target size for reduced microsatellite stability, resulting from failure of one or more of the steps shown in the figure, could be quite large. In *E. coli*, there are about 20 known mutator genes, and there is no reason to think that fewer genes control stability in humans. It will be interesting to see if the locus predisposing humans to colon cancer³ contains a gene for heteroduplex repair, and how many other instability loci are found. If many genes are involved in microsatellite instability there may be heterogeneity in the extent and the specificity of microsatellite instability in different populations and diseases.

The new work¹ raises a number of

1. Strand, M., Prolla, T. A., Liskay, R. M. & Petes, T. D. *Nature* **365**, 274–277 (1993).
2. Beckman, J. S. & Weber, J. L. *Genomics* **12**, 627–631 (1992).
3. Peltomäki, P. *et al.* *Science* **260**, 810–812 (1993).
4. Kuhl, D. A. & Caskey, C. T. *Curr. Opin. Genet. Devel.* **3**, 404–407 (1993).
5. Streisinger, G. *et al.* *Cold Spring Harb. Symp. quant. Biol.* **31**, 77–84 (1966).
6. Schaaper, R. M. & Radman, M. *EMBO J.* **8**, 3511–3516 (1989).
7. Modrich, P. *A. Rev. Genet.* **25**, 229–253 (1991).
8. Kunkel, T. A. *Biochemistry* **29**, 8003–8011 (1990).
9. Sinden, R. R. & Wells, R. D. *Curr. Opin. Biotech.* **3**, 612–622 (1993).
10. Loeb, L. A. *Cancer Res.* **51**, 3074–3079 (1991).

An enigma orbiting a puzzle

William B. McKinnon

AT the edge of our planetary system lie the planet Pluto and its large moon, Charon. Silently orbiting each other in eternal twilight (the Sun is more than a thousand times dimmer than at the Earth), each world hangs motionless in the other's sky, the end result of their tidal evolution. How large are these worlds, what are they made of, and where did they come from? These questions have fascinated since Pluto was discovered in 1930. Pluto is the one planet yet unvisited by spacecraft, so at a recent meeting* held in the city of the planet's discovery, planetary astronomers dominated. New data and interpretations were poured out for several days. Whatever the topic — size, mass, atmospheric structure, surface temperature, composition or origin — there were two data sets or at least two conflicting interpretations of the data, and understanding one aspect of the planet often hinged on understanding another. It was as if the Pluto–Charon binary were being deliberately elusive.

The diameters of Pluto and Charon have usually been considered settled since the two bodies went through a sequence of mutual occultations and transits between 1985 and 1990. These 'mutual events' yielded radii of $1,150 \pm 7$ and 593 ± 10 km for Pluto and Charon, respectively¹. Observations of an occultation of a star in 1988 proved that Pluto has an atmosphere, though, and this has bedevilled attempts to determine accurate radii for the bodies ever since². Both an analysis of all the stellar occultation data taken by various observatories (R. Millis, Lowell Observatory) and modelling of an independent set of mutual event data (E. Young, Massachusetts Institute of Technology) argued for a Pluto near 1,200 km radius or larger. These results bolster the case for a larger Pluto based on subsets of the stellar occultation data (for example, in ref. 3), but D. Tholen (Univ. of Hawaii) and M. Buie (Lowell) stood by their extensive mutual event analyses¹.

Interpretation of the stellar occultation curves, on which the argument for a bigger Pluto rests, is not straightforward. There are pronounced 'bites' in the profile taken by the Kuiper Airborne Observatory, which may represent extinction by a haze layer or refraction due to a temperature gradient in the atmosphere, or both^{2,3}. Theoretical arguments against a substantial aerosol haze exist, but Neptune's satellite Triton, considered to be Pluto's

planetary cousin (Fig. 1), shows hazes and low-altitude clouds (R. Yelle, Univ. of Arizona), so Pluto may as well. On the other hand, Pluto's extended atmosphere has a temperature near 100 K, which is substantially higher than estimates of its surface temperature. So at some level a temperature gradient, increasing with altitude, must exist. Just to complicate the story further, there is also a possibility of a near-surface troposphere (J. Stansberry, Univ. of Arizona), implying a tempera-



Two arguably similar rock–ice worlds of the deep outer Solar System, Neptune's Triton (left), 1,350 km in diameter, as seen by Voyager 2, and to scale, Pluto and Charon (right), with regional albedos and global colours determined during the recent series of mutual occultations and transits (image courtesy of P. Schenk, Lunar and Planetary Institute).

ture decreasing with altitude.

The determination of Pluto's atmospheric temperature is itself a result of the stellar occultation, but what the occultation actually provides is a measure of T/μ , temperature over molecular weight. To know the true temperature, one must know the atmospheric composition. This was uncertain until recently, when nitrogen ice was detected as the dominant component of Pluto's surface (D. Cruikshank, NASA Ames; ref. 4). It is nitrogen gas then, in vapour pressure equilibrium with the surface ice, that forms Pluto's atmosphere (and Triton's as well, for that matter). A nitrogen atmosphere yields $T \approx 100$ K, a value consistent with heating and cooling controlled by methane vapour⁵, but the infrared spectra of Pluto show that methane is a minor component, about one per cent, dissolved in the nitrogen ice, and unable to contribute to Pluto's atmosphere because of its low volatility and dissolution⁴. So why is Pluto's atmosphere acting as if it were part methane (0.1 per cent or greater is required)? Could haze particles provide the heating? Are volcanic eruptions or Triton-like plumes spewing methane into space?

A nitrogen atmosphere also runs into trouble if the temperature determined by the Infrared Astronomical Satellite

(IRAS) for Pluto, 55–60 K (ref. 6), is accepted. In vapour pressure equilibrium, the surface pressure implied would be a few tens of millibar, far too great to reconcile with the stellar occultation observations.

Millimetre-wavelength radio observations (D. Weintraub, Vanderbilt Univ.) indicate a much cooler Pluto, 30–44 K. Such surface temperatures can yield just the right atmospheric thickness, but there is no easy reconciliation of the IRAS and millimetre-radio results. Wavelength-dependent emissivity effects may be the answer; it has long been known, for example, that the microwave emissivities of the icy jovian satellites Ganymede and Europa are substantially less than unity, which is manifested as lower brightness temperatures⁷. Their surfaces, however, are water ice at a specific temperature range, not nitrogen ice at another. Perhaps Pluto is simply geologically complex, with large regions of different compositions and temperatures. The planet is known to possess bright polar and darker equatorial regions¹; the albedo variation is the strongest of any icy world in the outer Solar System save Saturn's Iapetus.

The interlocking puzzle of Pluto's atmosphere, temperature, surface and size is most important for understanding the planet's composition and hence its origin. Composition refers to

the surface, where the most volatile materials accreted may be found, and to the bulk of the planet (and satellite), usually discussed in terms of the calculated ratio of water ice to rock⁸. Both are diagnostic of conditions of formation and subsequent evolution. It seems that as far as Pluto is concerned "bigger is better" (J. Lunine, Univ. of Arizona; and W.B.M.). That is, the somewhat larger diameter for Pluto above, coupled with the known mass of the Pluto–Charon system, yields a system density closer to that predicted by the latest models of outer solar nebula chemistry⁹. Such a theoretical match, however, takes no account of the complications that ensue if a substantial fraction of Pluto is organic matter, as is the case in comets such as Halley (to which Pluto may be related).

Charon, the satellite, remains an enigma. All we know for certain are its orbit, its approximate size, and that there is water ice on its surface and very little if any atmosphere². A recent measurement of Pluto's barycentric wobble implies that Charon itself is ice-rich¹⁰, but preliminary ground-based astrometry (L. Young, Massachusetts Institute of Technology) points to a Pluto and Charon of equal density. An accurate value of their mass ratio is necessary to determine the angular

*Pluto & Charon, Flagstaff, Arizona, 6–9 July 1993.

momentum of the system, which would constrain the origin of the binary (such as via the collision of two precursors). Even the pronunciation of the satellite's name is disputed (both 'car on' and 'share on' are in use).

The origin of Pluto itself may be coming into focus, however. Recent calculations of test particles in circular coplanar orbits beyond the giant planets show that because of gravitational perturbations the regions out to about 45 astronomical units (AU; the distance between the Earth and Sun) are cleared out over several 100 million years or longer¹¹. Near 40 AU, the position of the 3:2 orbital period resonance with Neptune, particles are perturbed into orbits of high eccentricity and high inclination very similar to that of Pluto and Charon, before ejection. All that is necessary to make such orbits permanently stable, and completely Pluto-like, is the nudge supplied by a substantial impact (H. Levison and A. Stern, Southwest Research Institute). And direct capture into the 3:2 resonance that Pluto occupies may be possible if Neptune slowly evolved outwards from the Sun (which it may have done as it scattered planetesimals) (R. Malhotra, Lunar and Planetary Institute). If so, collision would not be necessary (although they are unavoidable).

Thus Pluto turns out to be a critically important object, a large rock-ice world, and planet by convention, born at the boundary between the realm of the gas giants and the Kuiper belt of comets beyond. It is probably also relatively unprocessed, at least compared with Triton, which may have been severely tidally heated after its capture by Neptune. It awaits detailed inspection by a planetary mission such as NASA's contemplated Pluto Fast Flyby, but may yet escape examination. It passed perihelion inside Neptune's orbit a few years back, will regain its status as the ninth planet in March 1999, and then sail into the black reaches of space beyond Neptune. □

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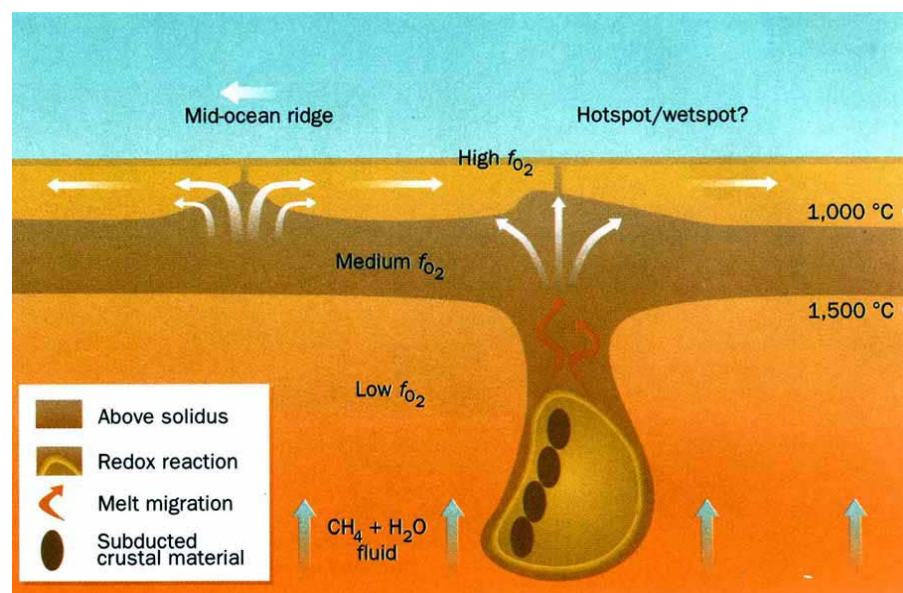
The other carbon cycle

D. H. Green, S. M. Eggins and G. Yaxley

THE 'carbon cycle' is generally taken to refer to the Earth's atmosphere, which contains about 750 billion tonnes of carbon. But geologists see a far larger carbon cycle — the carbon locked up in carbonate minerals in upper crustal rocks is estimated to be 7.5×10^{14} tonnes. An important question is whether significant amounts of carbonate are returned into the mantle by subduction. On page 221 of

infiltration and reaction with the oceanic lithosphere².

The EMII signature is indicative of recycled sediments; HIMU is indicative of subducted oceanic crust. This EMII signature is the least 'diluted' form yet recovered from mantle materials. In demonstrating that carbonatite imprints the EMII and HIMU signatures in the mantle, the authors show how essential it is to



Melting anomaly from redox reactions around subducted slab. Here there is a fixed 'hotspot' and drifting mid-ocean ridge with lithosphere above asthenosphere containing incipient melt. In this scheme, the melting anomaly results not from unusually high temperatures but from redox reactions around a subducted slab of material whose oxygen fugacity f_{O_2} is higher than that of the normal mantle. Oxidized conditions and the presence of H_2O plus C or H_2O plus CO_2 greatly lower the solidus at depths below the asthenosphere⁹.

this issue, Hauri and co-workers provide new insight on this and several other problems into the bargain¹.

Their results come from a study of stray fragments (xenoliths) of oceanic lithosphere found in basalts from two ocean islands. The harzburgite and lherzolite xenoliths have reacted in the mantle with carbonate-rich (carbonatite) melts, which have introduced distinctive isotopic and trace-element fingerprints. Four of the xenoliths show extreme isotope signatures: in two, from Savai'i in Samoa, the isotope ratios of the radiogenic elements strontium, lead and neodymium are of the 'enriched mantle II' (EMII) character, and in another two, from Tubuai in the Austral Islands, they are characteristic of the 'HIMU' mantle composition. The host basalts of these islands show much less extreme isotopic compositions, spread between these and other mantle endmembers. Hauri and collaborators draw a link between the different HIMU and EMII signatures and the process of carbonatite

consider complex effects of different solidi on mantle chemical and physical dynamics. To do this requires a knowledge of oxygen fugacity (f_{O_2}) in the mantle³, as there are multiple solidi, each appropriate for a particular carbon and hydrogen concentration and f_{O_2} . If the oxygen fugacity in the upper mantle is laterally as well as vertically inhomogeneous, then the solidus and chemical character of melting will vary (see figure). And because of their extreme fluxing effects in mantle melting, what seem to be tiny concentrations of carbon and hydrogen (50 to 500 p.p.m. C, 0.02–0.2 weight per cent H_2O) have a huge effect on upper-mantle behaviour.

The authors note that "some mechanism must transport CO_2 , and probably other volatiles as well, through subduction zones and into the convecting mantle". There is other evidence for this from the concentrations and isotopic signatures of gaseous species in basalts, in mantle xenoliths and their fluid inclusions. The study

1. Buie, M. W., Tholen, D. J. & Horne, K. *Icarus* **97**, 211–227 (1992).
2. Stern, S. A. *Rev. Astr. Astrophys.* **30**, 185–233 (1992).
3. Eliot, J. L. & Young, L. A. *Astr. J.* **103**, 991–1015 (1992).
4. Owen, T. C. *et al.* *Science* **261**, 745–748 (1993).
5. Yelle, R. V. & Lunine, J. I. *Nature* **339**, 288–290 (1989).
6. Sykes, M. V., Cutri, R. M., Lebofsky, L. A. & Binzel, R. P. *Science* **237**, 1336–1340 (1987).
7. Muhleman, D. O. & Berge, G. L. *Icarus* **92**, 263–272 (1991).
8. McKinnon, W. B. & Mueller, S. *Nature* **335**, 240–243 (1988).
9. Lunine, J. I. *Science* **261**, 697–698 (1993).
10. Null, G. W., Owen, M. & Synnott, S. P. *Astr. J.* **105**, 2319–2335 (1993).
11. Holman, M. J. & Wisdom, J. *Astr. J.* **105**, 1987–1999 (1993).

of carbon and nitrogen led Zhang and Zindler⁴ to conclude that about 72 per cent of the total degassable CO₂ (and 12 per cent of the nitrogen) from the primitive Earth is still present in the mantle, strongly suggesting to them that most CO₂ has been recycled by subduction. Trull *et al.*⁵, who looked at trends in carbon and helium isotope ratios for mid-ocean-ridge and 'hotspot' sampling of the upper mantle, argue that the upper mantle reservoir (which supplies the mid-ocean-ridge basalts) does not require significant carbon recycling but that the 'hotspot' source, often equated with the lower mantle (in part because of the associated higher ³He/⁴He ratios), does contain recycled carbon.

In conventional models of subduction, fluids containing carbon, hydrogen and oxygen are extensively degassed by metamorphic reactions during subduction. Nevertheless, there have been cases where eclogites (subducted material) have been found to contain primary carbonates, and there are even marbles associated with eclogites that record subduction paths to pressures of 2–3 GPa (depths of 70–100 km) before return to surface conditions⁶. And experimental work⁷ shows that when garnet amphibolite transforms to eclogite at subduction pressures and temperatures, any carbonate present remains behind in the solid residue from a low-temperature, hydrous silicate melt (700–1,000 °C, pressures above 2.5 GPa). So carbonate-bearing eclogites could be subducted into the mantle as refractory residues. Recent evidence for carbonate and CO₂ inclusions within diamond may support this⁸.

If normal, deep mantle is at a 'low' oxygen fugacity of IW (iron-wustite) + 1 log unit, then it will be below its solidus (melting temperature) and carbon and hydrogen present will be mobile in a CH₄ + H₂O fluid. Subducted lithosphere carrying refractory carbonate-bearing eclogite will also be subsolidus but will have a higher oxygen fugacity ($f_{O_2} \geq IW + 3$ log units) (see figure). The redox contrast between normal mantle and subducted slab is a potential focus for reaction. If the mantle is still degassing carbon and hydrogen species along with primitive noble gas signatures, then a CH₄ + H₂O carrier fluid may react on penetrating the

subducted material, reducing carbonated eclogite to diamond-bearing eclogite and H₂O-rich fluid, and triggering water-rich silicate melting. So the fact that a 'lower-mantle' helium isotopic signature is associated with evidence for carbon recycling may be a consequence of the redox reaction of subducted crustal carbonate and deep-mantle CH₄-rich fluid. Consequent silicate melts at the redox interfaces will reflect the character of crustal components mixed into the upper mantle, at either shallow or deep (transition zone) levels (see figure).

In 'hotspot' basalt petrogenesis, a higher volatile (CO₂ + H₂O) content in primitive melts suggests that the mantle melting anomaly may have its deep-seated cause in redox interactions and volatile-fluxed melting (see figure). Volatile-fluxed melting at interfaces between subducted, oxidized lithosphere and normal mantle may explain the apparent mixing of different isotopic components in 'hotspot' basalts, that is, oceanic crust and carbonate signatures, deep mantle

degassing signatures and normal mantle signatures. Insofar as geophysical interpretations indicate anomalous mantle characteristics at asthenospheric depths beneath hotspots, these may be interpretable in terms of volatile-induced melting rather than very large temperature anomalies.

It is a large extrapolation from isotopic measurements on a few mantle fragments to the major aspects of mantle dynamics. But the effects of carbon and hydrogen in the upper mantle are first-order controls on fluid presence or absence and on melting, and thus, by inference, on rheological properties and mantle dynamics. Hauri *et al.* may have provided a critical linking piece in the jigsaw of mantle dynamics. □

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ANTIGEN PROCESSING

Peptides and the proteasome

Jonathan C. Howard and Angela Seelig

THANKS to the efforts of many scientists, as interpreted by an almost equal number of News and Views writers ever since Parham¹, we have come to an accessible view of the origin of the cytosolic peptides with which class I molecules of the major histocompatibility complex (MHC) are designed to assemble. Three papers^{2–4}, two of which appear on pages 262 and 264 of this issue^{2,3}, now threaten to clarify matters further. They draw attention, once again, to the involvement of particular MHC-encoded components of a cytosolic protease, the proteasome, in the process.

The accessible view looks like this. Proteins originating in the cell by endogenous biosynthesis are cleaved by cytosolic proteases. Peptide fragments are then transported out of the cytosol into the lumen of the endoplasmic reticulum by an ATP-dependent MHC-encoded peptide transporter (TAP) localized to this membrane boundary. There they assemble into nascent class I MHC molecules immediately after synthesis, causing the release of the ternary complex (heavy chain, β_2 -microglobulin and peptide) from a swarm of chaperones and the eventual expression of the class I molecule loaded with peptide on the cell surface. Here the peptides, partially exposed in the binding cleft of the MHC molecule, are available for specific recognition by T-cell receptors.

The identity of the usual protease

associated with class I antigen processing is now becoming the centre of interest and, as with TAP, there is an obvious candidate — namely the proteasome, an abundant multi-subunit cytosolic protease. The only cellular function unambiguously associated with the proteasome is the ATP-dependent degradation of protein substrates conjugated to ubiquitin. This pathway is known to be involved in degradation of short-lived and abnormally folded proteins in normal cells⁵, and has twice been implicated by elegant experiments in the provision of antigenic peptides for class I assembly^{6,7}.

Distinctive role

The proteasome was implicated again in antigen processing when it was discovered that two subunits, known as LMP2 and LMP7, are coded in the MHC, interdigitating the two chains of the peptide transporter, TAP. Furthermore, both LMP2 and LMP7 are stimulated by γ -interferon like the TAP genes and the MHC molecules themselves (though to different extents in different cell types, and probably also under different culture conditions). These results suggested that the MHC-encoded proteasome chains would play some distinctive role in protein degradation for antigen processing. Last year, however, two reports^{8,9} showed that MHC mutant cell lines with defective class I expression, and lacking genes for both TAP and LMP2/LMP7, could be restored

1. Hauri, E. H., Shimizu, N., Dieu, J. J. & Hart, S. R. *Nature* **365**, 221–227 (1993).
2. Yaxley, G. M., Crawford, A. J. & Green, D. H. *Earth planet. Sci. Lett.* **107**, 305–317 (1991).
3. Bailhaus, C. F., Berry, R. F. & Green, D. H. *Contrib. Mineral. Petrol.* **107**, 27–40 (1991).
4. Zhang, Y. & Zindler, A. *Earth planet. Sci. Lett.* **117**, 331–345 (1993).
5. Trull, T., Nadeau, S., Pineau, F., Polvé, M. & Javoy, M. *Earth planet. Sci. Lett.* **118**, 43–64 (1993).
6. Becker, H. & Altherr, R. *Nature* **358**, 745–748 (1992).
7. Yaxley, G. M. thesis, Univ. of Tasmania (1993).
8. Schrauder, M. & Navon, O. *Nature* **365**, 42–44 (1993).
9. Green, D. H., Falloon, T. J. & Taylor, W. R. in *Magmatic Processes: Physico-Chemical Principles* (ed. Mysen, B. O.) 139–154 (Geochem. Soc. Spec. Publ. 1, 1987).

to normal class I antigen expression and presentation merely by the replacement of the missing TAP subunits. The genes encoding LMP2/LMP7 did not appear to be required for antigen processing⁸; indeed the entire complex pattern of peptides loaded into class I MHC molecules in resting cells seemed unaffected either qualitatively or quantitatively by the lack of the two MHC-linked proteasome subunits⁹.

The new studies²⁻⁴ bring the LMP2/LMP7 chains back into consideration by showing that their presence or absence is associated with subtle changes of emphasis in the preferred cleavage specificity of the proteasome. All three groups used short fluorogenic oligopeptide substrates to test the cleavage specificity of proteasomes prepared from tissue culture cells. Driscoll *et al.*² and Gaczynska *et al.*³ worked with human hepatoma and macrophage cell lines, either resting or stimulated with γ -interferon, and lymphoblastoid cells carrying or lacking LMP2/LMP7 subunits genetically. They agree that the main change in specificity associated with expression of LMP2/LMP7 is an increase in the rate of cleavages leaving positively charged or hydrophobic carboxy-terminal (P1) amino acids. Gaczynska *et al.* also report a reduction in cleavages generating negatively charged carboxy termini not seen by Driscoll and colleagues.

Discrepancies

Boes *et al.*⁴, who worked on resting and γ -interferon-stimulated mouse fibroblasts, report a rather different result. They find a reduction in the efficiency of cleavages generating hydrophobic carboxy termini associated with the induction of the LMP2 chain (the LMP7 chain is constitutively expressed in the transformed fibroblasts used in this study), and no change in cleavages generating positively charged carboxy termini. It is too early to interpret the apparent discrepancies between the three sets of results, except to note the differences in species, cell type, protease preparation and substrates used.

The study of proteasome cleavage specificity has been overly influenced by previous experience with well-defined proteases such as trypsin and chymotrypsin, and the importance of sequence environment in determining cleavage by the proteasome has not yet been extensively investigated. Boes *et al.* approach this point in their *in vitro* analysis of cleavage of a synthetic 25-mer oligopeptide derived from a murine cytomegalovirus protein. Interestingly, the cleavage specificity of proteasomes from cells treated with γ -interferon differs from that of resting proteasomes not by the nature of the P1 amino acid, but by which of two instances of a favoured P1 amino acid (in

this case a tyrosine) is used.

There is an additional agenda in all these studies, namely, that the inclusion of the LMP2/LMP7 chain in the proteasome is good for antigen processing in some way. Driscoll *et al.* and Gaczynska *et al.* both emphasize that the carboxy-terminal amino acids of peptides actually loaded into class I MHC molecules are nearly always either positively charged or hydrophobic¹⁰. Thus the incorporation of LMP2/LMP7 into the proteasome would favour the generation of 'loadable' carboxy termini, and is at least consistent with the idea that proteasomes are responsible for creating the observed carboxy termini of naturally loaded peptides. The same argument appears not to hold for the amino termini where there is no consistency in the implied P1 amino acid (knowing the protein sequence from which they are derived), suggesting that the amino termini of loaded peptides are generated by a distinct proteolytic activity. In the same spirit, Boes *et al.* point out that the interferon-induced shift in preferred cleavage of their 25-mer would favour the release of a known 9-mer epitope.

The underlying issue is how typical class I peptides, which are normally 8–10 amino acids in length, are generated in their final form. Is it by proteolytic activity in the cytosol, followed by transport and loading? Or do they arise in two steps? That is, by a cytosolic step generating (on average) long peptides with hydrophobic or positively charged carboxy termini, followed by transport to the endoplasmic reticulum, assembly with the carboxy terminus correctly positioned in the peptide-binding groove, and amino-terminal trimming to optimal size, as proposed by Rammensee and colleagues¹¹. Some of the present data are at least consistent with this second notion. But there is still a grave shortage of relevant information, not least concerning the potentially selective properties of the peptide transporter^{12,13}. □

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1. Parham, P. *Nature* **348**, 674–675 (1990).
2. Driscoll, J., Brown, M. G. & Monaco, J. J. *Nature* **365**, 262–264 (1993).
3. Gaczynska, M., Rock, K. L. & Goldberg, A. L. *Nature* **365**, 264–267 (1993).
4. Boes, B. *et al.* *J. exp. Med.* (in the press).
5. Goldberg, A. L. & Rock, K. L. *Nature* **357**, 375–379 (1992).
6. Townsend, A. *et al.* *J. exp. Med.* **168**, 1211–1224 (1988).
7. Michalek, M. T., Grant, E. P., Gramm, C., Goldberg, A. L. & Rock, K. L. *Nature* **363**, 552–554 (1993).
8. Momburg, F. *et al.* *Nature* **360**, 174–177 (1992).
9. Arnold, D. *et al.* *Nature* **360**, 171–173 (1992).
10. Falk, K. & Röttschke, O. *Seminars Immun.* **5**, 81 (1993).
11. Rammensee, H.-G., Falk, K. & Röttschke, O. *Rev. Immun.* **11**, 213–244 (1993).
12. Shepherd, J. C. *et al.* *Cell* **74**, 577–584 (1993).
13. Neefjes, J. J., Momburg, F. & Hammerling, G. J. *Science* **261**, 769–771 (1993).

Smoothed air

A SUBSONIC aircraft is designed to slip through the air with minimum drag. A warning pressure wave travels in front of it, diverging the air ahead slightly to let it through. But a supersonic aircraft cannot exploit this effect, for it travels faster than its own pressure wave. It just slams into static, unwarmed air, kicking it aside as a conical shock wave which runs off with a vast amount of power, and hits the ground as a supersonic boom.

Daedalus has a way out. Suppose, he says, the aircraft projected ahead of it a contoured beam of some radiation absorbed by air. This could warm up the air ahead, and start it expanding out of the way of the approaching craft. By the time the craft arrived, the air ahead of it could be moving out of its way at just the right speed to accept the advancing shape quietly, radiating no energy as shock waves. Power would be needed to generate the radiation beam, but this would be saved by the vastly reduced load on the engines. In the ultimate limit, so much energy could be pumped into the air ahead that it would explode right out of the way of the advancing plane. The craft would then coast along in a continuously generated tunnel of vacuum, and would need no engine power at all. This, however, would probably consume more power in radiation than would be needed by the thirstiest conventional engines. Somewhere between these extremes lies the optimal minimum-power design.

Determining that design will pose the most complex and subtle mathematical problems. The aircraft and its advance radiation envelope have to match each other perfectly at cruising supersonic speed; in addition, the craft has to retain reasonable lift and stability in subsonic flight. Even Daedalus has no idea what the final result will look like. He is not even sure of the best radiation to use. Ion beams, some microwave and far infrared frequencies, and far ultraviolet are all absorbed by air. Far infrared, for which powerful laser sources are available, is probably the most immediately feasible choice.

The final technology should transform aviation. Aircraft designers will rush to redevelop the glittering supersonic, hypersonic, aerospace and ramjet vehicles stalled these past 20 years by high oil prices and supersonic-boom restrictions. Politicians and businessmen will flash round the world in a technomaniac dream, saving precious hours in which to savour at their destinations the leisurely, unchanging realities of baggage mishandling, customs and immigration formalities, traffic jams, and jet-lag. David Jones

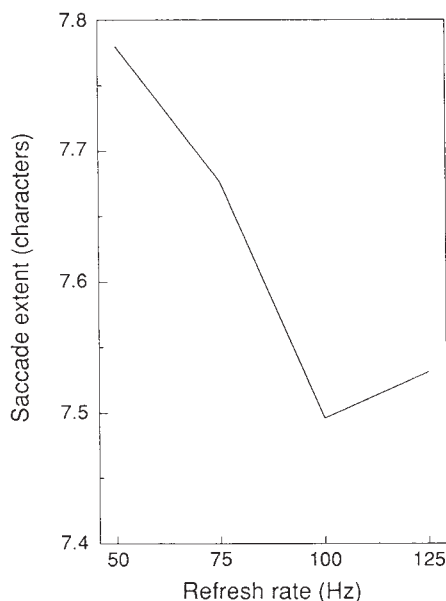
'Flicker' on VDU screens

SIR — There has long been widespread concern about possible adverse side-effects arising from the use of refreshed-screen computer displays. An EC directive has recently been implemented in UK law in the form of Health and Safety Regulations governing the use of display screen equipment¹. These regulations make specific reference to the need for the screen image to be "stable, with no flickering or other forms of instability". For a given phosphor, increases in the phenomenal stability of a raster display can be achieved by increasing refresh rate. However, we have evidence that disturbances to eye movement control occur at, or above, refresh rates of 100 Hz, well beyond the point at which users report a display as appearing stable. Furthermore, there are grounds for predicting that the obtained effect (a systematic change in average saccade extent) will impair reading performance. We have now demonstrated such a performance decrement associ-

ated with supra-threshold pulsation in a laboratory task.

We measured parameters of eye movement control as a sample of 24 student volunteers processed a string of three words (the word 'looks' or 'means' followed by two target words) presented on a variable-frequency refreshed screen display. The task mimicked the dynamics of normal reading by producing a short train of saccades directed towards and across the target words. Depending on the initial word, subjects were asked to indicate, by pressing buttons, whether the two targets had identical spelling or similar meaning. Four refresh rates were used (50, 75, 100 and 125 Hz) with brightness carefully controlled. Word length and spacing were also manipulated. The primary measure was the size of the first saccade entering each of the two target words. Launch position for the first saccade was predetermined and for the second depended only on measurable changes, if any, in location within the first target word (refixations). Saccade extent into both words decreased with increasing refresh rate. The effect was statistically reliable in the case of saccades directed towards word two (see figure), with a reduction in average saccade extent of a third of a character.

Small changes in the size of the first saccade directed towards a word have a disproportionate effect on reading performance because, for a word of given length, there is an optimal inspection point² and deviations from this, even by



Extent of first saccade entering the second target word as a function of refresh rate. The following factors entered into an initial analysis of variance: Task (identity of spelling versus meaning); spacing (1 versus 3 characters); target word (1 versus 2); refresh rate (four levels: 50, 75, 100 and 125 Hz); and word length (4, 5, 7 and 8 letters). There was a significant interaction between spacing, word and refresh rate, $F = 2.86$, d.f. 3/60, $P = 0.044$. Simple main effects analyses showed no significant effect for word 1, but a significant effect of refresh rate for word 2, $F = 3.44$, d.f. 2/49, $P = 0.039$. (Probabilities given follow application of the Greenhouse-Geisser adjustment.) Tests on individual contrasts using the Newman-Keuls procedure show a significant reduction in saccade extent between 50 and 100 Hz ($P = 0.031$) and between 50 and 125 Hz ($P = 0.042$).

only one or two letters, exact a penalty in the form of increased processing time, mostly stemming from changes in numbers of within-word refixations³. Consistent with this, our data also show a statistically significant increase in the probability of right-going within-word refixations as a function of refresh rate.

Other psychophysical evidence suggests that supra-threshold pulsation influences visual stability^{4,5}. Our results suggest an adverse influence on reading performance, with a continuing decrement up to an asymptote at about 100 Hz (this occurs in addition to the known effects of relatively low-frequency flicker on performance⁶). It is likely that the increased number of small corrective saccades induced by high-frequency pulsation exacts a penalty in terms of increased attentional demand and contributes to reports of visual fatigue. If so, seeking a solution to the general problem of adverse effects associated with refreshed screen displays by increasing refresh rate may well be inappropriate.

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1. Health and Safety Executive (UK). *Guidance on Health and Safety (Display Screen Equipment) Regulations, 1992* (HMSO, London, 1992).
2. O'Regan, J. K. & Levy-Schoen, A. in *Attention and Performance XII: The Psychology of Reading* (ed. Coltheart, M.) 363–383 (Erlbaum, Hillsdale, New Jersey, 1987).
3. McConkie, G. W., Kerr, P. W., Reddix, M. D., Zola, D. & Jacobs, A. M. *Percept. Psychophys.* **46**, 245–253 (1989).
4. Macnik, S. L., Fisher, B. D. & Bridgeman, B. *Vis. Res.* **31**, 2057–2064 (1991).
5. Hershberger, W. *Percept. Psychophys.* **41**, 35–44 (1987).
6. Wilkins, A. J. *Hum. Fact.* **28**, 75–81 (1986).

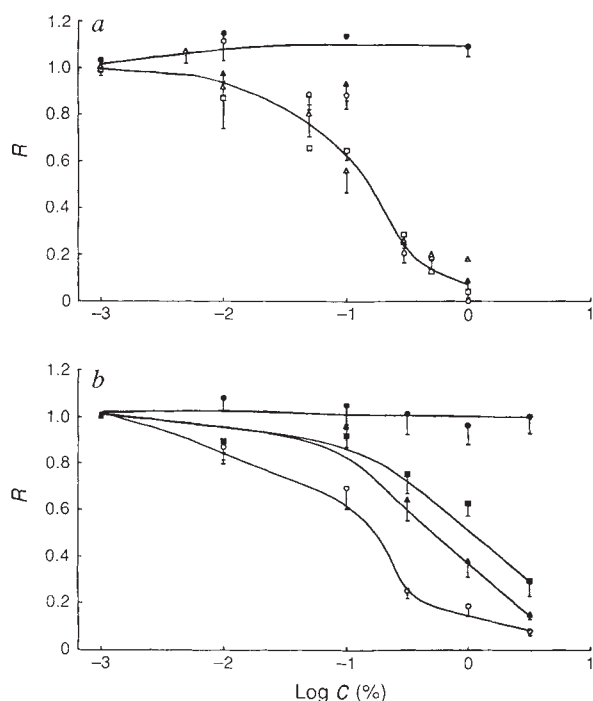
Specific inhibitor for bitter taste

SIR — A way to mask bitter tastes is a desirable goal for food and pharmaceutical scientists as well as those interested in receptor physiology. A specific inhibitor for a bitter taste is useful for this purpose, but no such inhibitor has so far been available. Bitter substances are generally hydrophobic (T. Kumazawa *et al.* *Biochemistry* **27**, 1239; 1988); hence there may be hydrophobic substances which mask the receptor sites for bitter substances. We have found that a lipoprotein (PA-LG) made of phosphatidic acid (PA) and β -lactoglobulin (LG) completely suppresses taste responses in frogs and taste sensation in humans to all the bitter substances examined without affecting the responses to other taste stimuli. Both PA and LG are obtained from foods, being prepared from soya bean and milk, respectively. Hence this chemical can be safely used to mask the bitter taste of foods and drugs.

We prepared the lipoprotein PA-LG by homogenizing a suspension of PA and LG

in water and dehydrating the homogenate. The powder is easily dissolved in water, whereas PA alone is not. First we examined the effects of PA-LG on the taste responses by measuring the activities of the glossopharyngeal nerve. To eliminate the chance that PA-LG directly interacts with bitter substances, we pre-treated the tongue with 0.3% PA-LG for 10 min and then stimulated it by bitter substances dissolved in water. The responses to all eight bitter substances examined were greatly suppressed by PA-LG, whereas the responses to NaCl, acetic acid, L-alanine and galactose were not. We also examined the effects of lipoproteins made of LG and other lipids such as phosphatidylcholine, triacylglycerol and diacylglycerol on responses to the bitter substances, but none had a marked effect.

The relative magnitudes of the taste nerve responses in frogs to quinine hydrochloride, L-leucine, caffeine, papaverine hydrochloride and NaCl as a function of PA-LG concentrations are shown in the



Effects of PA-LG on the taste nerve responses in frogs (a) and taste sensation in humans (b) to quinine hydrochloride ($\circ$), L-leucine ($\square$), caffeine ($\triangle$) and papaverine hydrochloride ($\blacktriangle$), L-isoleucine ($\blacksquare$) and NaCl ($\bullet$). R in the ordinate represents relative magnitude of taste sensitivity.

figure (a). Responses to bitter substances are decreased with increased concentration of PA-LG to a level of 1 per cent, while the response to NaCl is unaffected by all concentrations of PA-LG examined. These results indicate that high concentrations of PA-LG completely suppress the bitter responses by full occupation of the receptor sites. The data obtained with four bitter substances roughly follow a single curve, suggesting

emphasize that PA-LG is useful for masking bitter tastes of drugs and foods, and also for exploring characteristics of the receptor sites for bitter taste.

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Death of an ice sheet

SIR — The Barbados and New Guinea records of sea-level history suggest that there were two massive glacial-meltwater inflows during the past 15,000 years¹⁻³. The timing of these inflows relative to other environmental changes (such as changes in North Atlantic deep water (NADW) circulation) figures prominently in efforts to understand the last glacial to interglacial climate transition and the Younger Dryas event⁴. According to prevailing oceanographic theory, the magnitude of the first inflow was large enough to have shut down NADW circulation, thereby initiating colder climates in Europe (for example the Younger Dryas event)⁵. We have conducted ice-sheet modelling experiments which suggest why there were two massive, but short-lived, inflows of glacial meltwater as opposed to a single, more gradual influx^{6,7}. In particular, our simulations identify the first meltwater inflow event (approximately

14,000 calendar years ago) with the collapse of the Eurasian ice sheet (EIS), a marine ice sheet that covered Scandinavia and the Barents, Kara and Laptev seas⁸.

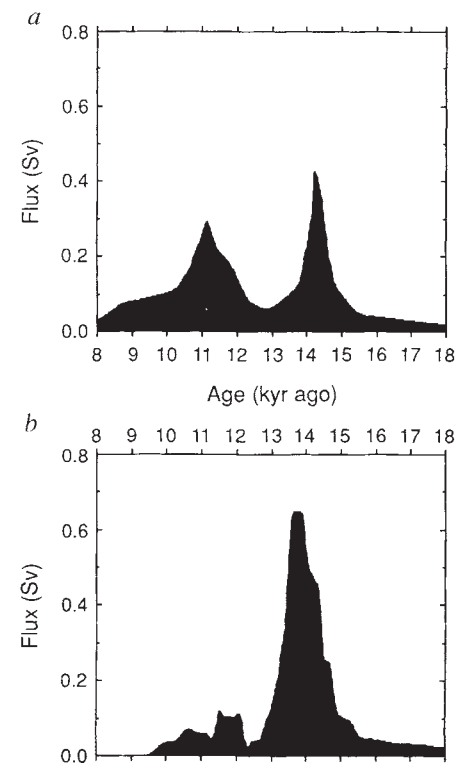
Our simulations determined a steady-state initial configuration of the EIS associated with estimated glacial-maximum climate. The modelled EIS was then forced to retreat according to a prescribed atmospheric-warming schedule indexed to a simplified atmospheric- CO_2 chronology derived from the Vostok ice core⁹. (The Vostok ice-core record and our assumptions concerning its relevance to climate conditions over the EIS determined the timing of EIS retreat in the simulation.) The figure compares the observed and simulated meltwater-inflow histories, Q_o and Q_m , respectively. Both histories display a peak that occurs approximately 14,000 calendar years ago. (Recent comparisons³ between the Bar-

bados and New Guinea sea-level records suggests that uncertainty in the timing of this peak is approximately 1,000 years.)

The peak in Q_m is larger (by approximately 50 per cent) and more recent (by 500 years) than that in Q_o . When we conducted our simulations, we knew nothing about the Barbados or New Guinea sea-level histories; thus, our simulations were not fine-tuned to reduce the volume of the initial EIS configuration needed for better agreement between Q_o and Q_m . Although our model did not address the Laurentide ice sheet (LIS), we infer that the second pulse in Q_o (at approximately 11,000 years ago) represents the demise of the LIS.

We attribute the rapidity of sea-level rise achieved by our model to the acceleration of ice-sheet flow towards icebergalving margins, rather than to a process of enhanced ice-sheet surface melting. This acceleration is caused by the retreat of grounding lines (boundaries between floating ice shelves and grounded ice sheets) into regions of isostatically depressed bedrock topography. Retreat into regions of lower bedrock elevation reduces friction which restrains seaward ice flow and, at the same time, increases (locally) the stresses which drive this flow. Ice-sheet mechanics may thus account for the unanticipated strength and brevity of the two meltwater pulses seen in the sea-level record.

Our model cannot explain why the demise of the LIS was delayed for 2,500



a, Observed history of meltwater influx¹⁻⁴; b, modelled history of meltwater influx due to EIS collapse^{6,7}.

years or so following the collapse of the EIS (see figure). Perhaps this delay was a result of transatlantic asymmetry in the

1. Fairbanks, R. G. *Nature* **342**, 637–642 (1989).
2. Bard, E., Hamelin, B., Fairbanks, R. G. & Zindler, A. *Nature* **345**, 405–410 (1990).
3. Edwards, R. L. *et al.* *Science* **260**, 962–968 (1993).
4. Charles, C. D. & Fairbanks, R. G. *Nature* **355**, 416–419 (1992).
5. Stocker, T. F. & Wright, D. G. *Nature* **351**, 729–732 (1991).
6. Lindstrom, D. R. *Paleoceanography* **5**, 207–227 (1990).
7. Lindstrom, D. R. & MacAyeal, D. R. *Science* **245**, 628–631 (1989).
8. Lehman, S. J. *et al.* *Nature* **349**, 513–516 (1991).
9. Barnola, J. M., Raynaud, D., Korotkevitch, T. S. & Lorius, C. *Nature* **329**, 408–413 (1987).

climate system, a higher tolerance of the LIS to climate warming, or the effects of short-lived cooling perturbations possibly caused by the first meltwater influx (such as Younger Dryas cooling).

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Recording fires by satellite

SIR — Cahoon *et al.*¹ illustrate the use of a satellite in mapping grass or forest fires at night. The wavelength band used was 0.4–1.1 μm , which spans the visible and very near infrared part of the spectrum. The illumination of cities was eliminated by their permanence, fires being of short duration. Only photographic prints, and not the basic digital information, were archived. The satellite used was one of the Defense Meteorological Satellite Program (DMSP) Block 5, with an effective resolution of about 2.7 km.

Routine National Oceanic and Atmospheric Administration (NOAA) meteorological satellites seem more suitable in principle for this task, for every part of the Earth is traversed twice every 24 hours, in midday sunshine and at night. The intensity of sunshine is a maximum at 0.5 μm (corresponding to a temperature of 6,000 K) and the Earth's emission has a maximum at about 11.0 μm (300 K). These two emission spectra cross over with an intensity of about one-fortieth of the maximum at a wavelength of about 4 μm , the precise values depending on the emission temperatures and other factors. The satellite messages are in archives at the University of Dundee and are generally available.

The NOAA satellites record the scene below them in several wavebands, one of which, channel 3, is 3.55–3.93 μm . This is about the maximum strength in the emission spectrum for a temperature of about 700 K, or dull red heat. Flames at 1,200–1,500 K are about 16 times as intense although in channel 3 the strength is around half the maximum and so they can

be 5 or 10 times as strong as red heat. But a flame with an area of only 20×20 m will outshine 1 km² of ordinary territory in bright sunshine and will be seen as very bright at night, while urban illumination is

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A pass by NOAA-9 at 13.46 GMT, 12 September 1985, as seen by the channel 3 waveband, which is also in a water vapour window. Fires in which surplus straw is being burned on the farms of England after the harvest can be seen. Other, more regular hotspots are also recorded, such as steel works in Dunfermline and Scunthorpe, and flares on North Sea platforms. But urban areas do not show up at night as they would in a visible waveband (see ref. 2).

undetected.

The figure shows the fires burning straw after the harvest on the farms of England in midday sunshine. Although channel 3 information was very often spoiled in the satellites before NOAA-7 by interference, later satellites have recorded it very well, and have been doing so for several years. A tape of the original digital message can usually give relative intensities, although big fires may saturate it.

That could easily be remedied if it were required for hotspot surveys; and the resolution (pixel size 1.1 km at nadir) is much better than DMSP records. However, to record the message at full resolution it would require the receiver to be within the general area being surveyed, for example one station at about the centre of gravity of Africa.

A more satisfactory method for equatorial observation is to use a geostationary satellite; and there is a reasonable prospect that the next Meteosat, above the Equator on the zero meridian, will have a recording channel in the same wavelength band as channel 3. The message could then be made available everywhere in Europe and Africa at any time of day or night, so it would be possible to make the most of cloud-free periods.

R. S. Scorer

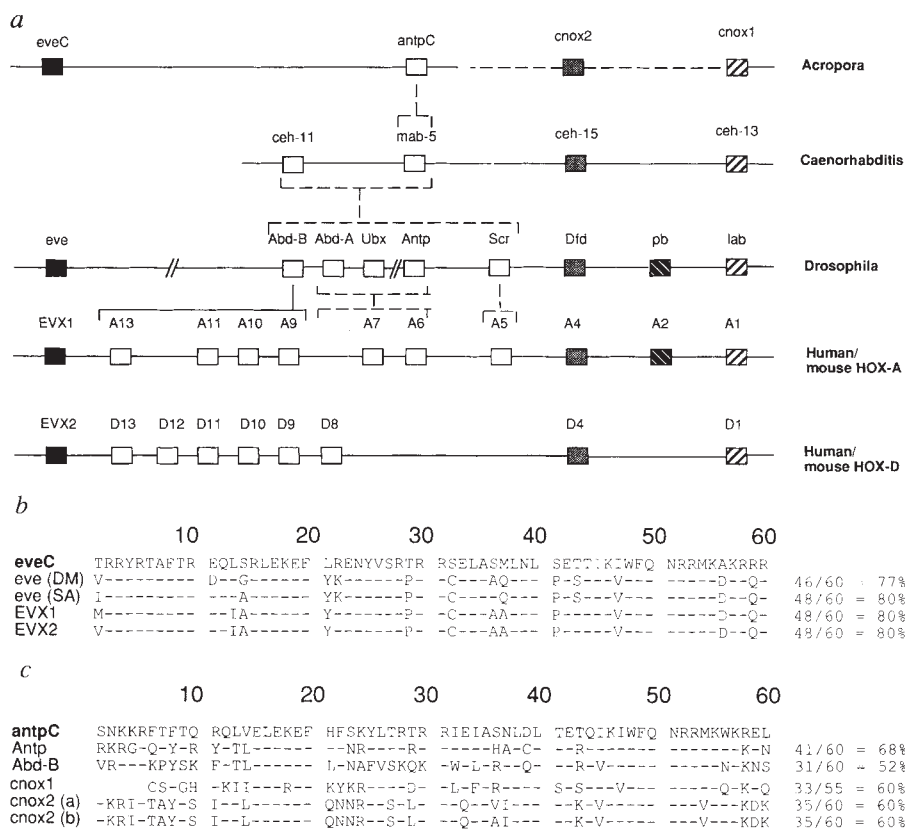
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1. Cahoon, D. R. *et al.* *Nature* **359**, 812–815 (1992).
2. Scorer, R. S. *Satellite as Microscope* (Ellis Horwood, Chichester, 1990).

Homeobox genes and the zootype

SIR — Possession of a specific set of genes involved in pattern formation has been proposed as the character defining the kingdom Animalia (the zootype¹). This concept arises from some remarkable similarities in terms of both structure and expression pattern between key development-regulating genes in relatively 'advanced' metazoans, specifically *Drosophila* and vertebrates. Comparative data for 'primitive' metazoans are required to validate the zootype concept and, as the most primitive metazoans with tissue-level organization, cnidarians are potentially one of the most informative 'lower' metazoan phyla. Here we demonstrate that *even-skipped* and *Antennapedia* class homeobox genes are adjacent in the genome of the coral *Acropora formosa*. This linkage in a cnidarian, observed also in vertebrates^{2,3} (a in the figure), suggests first that this is likely to be the ancestral arrangement of these genes; and second that the 'primitive' function of *even-skipped* class genes is in posterior pattern formation.

We have isolated a single *A. formosa* genomic clone containing a pair of homeobox genes. Subclones derived from one end of this genomic clone encode a homeodomain belonging to the *even-skipped* (*eve*) class⁴. The similarity between the predicted amino-acid sequence of the cnidarian *eve*-like homeodomain and its insect and vertebrate equivalents is, remarkably, approximately 77–80 per cent (b in the figure), showing that this class of genes was distinct very early in



a, Homology relationships between Hom/Hox clusters in the cnidarian *Acropora*, *C. elegans*, *D. melanogaster* and vertebrates. For simplicity, only two of the four vertebrate Hox clusters are shown. With the exception of the open boxes, pattern and shading indicate groups where clear homology relationships exist. Vertebrate subgroups 9–13 are *Abd-B* cognates, but homology relationships between other genes indicated by open boxes are less well established. The sequence and expression data for *cnox2* are consistent with this being the cnidarian cognate of *Deformed*^{5,6} and *cnox1* is thought to be a *labial* cognate⁵. In both *Drosophila* and vertebrates, there is a strong correlation between the position of a gene in the respective Hom/Hox cluster and spatial pattern of expression along the anterior/posterior body axis. **b**, Comparison of the *eveC* homeodomain with all other *eve*-class homeodomains. The human *EVX1* is identical with mouse *Evx-1* and *Xenopus Xhox-3* sequences, and mouse *Evx-2* is identical with *EVX2*. DM, *D. melanogaster*; SA, *Schistocerca americana*. **(c)** Comparison of *A. formosa Antennapedia*-class homeodomain with *Antp*, *Abd-B* and hydra sequences. Among the *Antp* class, the homeodomain sequences least related to *antpC* are *Abd-B* cognates (42–60 per cent identity). The *A. formosa* sequence has only 60 per cent sequence identity with the hydra *cnox1* and *cnox2* homeodomains, lower than its similarity to *Antp* cognates from distantly related organisms, implying that *antpC* belongs to another cognate group to those known from hydra. Note that the *cnox1* sequence is incomplete, and that there are *cnox2* sequences from two species.

metazoan evolution, and is good evidence for inclusion of *eve* in the definition of the zootype.

The second homeobox gene belongs to the *Antennapedia* (*Antp*) class, and is most similar to cognates of *Antp* (c in the figure). Significantly, the predicted amino-acid sequence of the *A. formosa* homeodomain differs more from *cnox1* and *cnox2*, *Antp*-class homeodomains previously identified from hydra^{5,6}, than from *Antp* cognates from representatives of other phyla (c). It is highly likely, therefore, that the *A. formosa Antp*-class homeodomain identified here corresponds to a different cognate group than those known from hydra. Because of its similarity with the *Drosophila* gene *Antp*, we refer to the *A. formosa Antp*-class gene as *antpC*.

In both *Drosophila* and vertebrates, all the known *Antp*-class homeobox genes

are clustered. This pattern of organization is thought to be very ancient, and to constitute a way of interpreting position along the anterior/posterior axis. In vertebrates, two *eve* homologues are present, both closely linked to the 5'-end of Hox gene clusters^{2,3}. However, *eve* is not linked to the Hom-C in *Drosophila*, and positional data are not yet available for other *eve*-class genes. This has led to speculation as to whether the organization in vertebrates reflects the ancestral pattern of organization or is a consequence of secondary gene capture. The proximity of the *eve* and *Antp*-class homeobox genes in *Acropora* clearly supports the former idea as they probably constitute the 5'-end of the cnidarian Hom/Hox-cluster, likely also to include the *Acropora* homologues of the hydra genes *cnox2* and *cnox1* (a in the figure), which are cognates of the *Drosophila* genes *Deformed* and *labial*,

respectively.

These results have other implications for the definition of the zootype. In vertebrates, the *Hox* genes proximal to *EVX1* and *EVX2* are both cognates of the *Drosophila* gene *Abd-B* (refs 2, 3). Thus, if the 'zootype' included an *Abd-B* cognate, then it should lie adjacent to *eveC* in the coral. In our analyses, however, of all the *Antennapedia* class genes, *antpC* was consistently least like cognates of *Abd-B*. This suggests that *Abd-B* may be more recent than predicted⁷, and that the Hom/Hox cluster in the ancestral metazoan consisted of fewer *Antp*-class genes than previously assumed.

We believe that the linkage of *eve* cognates to *Antp*-class genes is probably related to a 'primitive' function in posterior pattern formation. Thus, although *eve* early expression in *Drosophila* is in pair-rule stripes, in the grasshopper there is no sign of pair rule expression, with early expression being in the posterior mesoderm⁸, a pattern shared with the *Xenopus* and mouse *eve* homologues *Xhox-3* and *Evx-1*. Later in development the *eve* homologues in all of these organisms are expressed in the nervous system. These shared expression patterns suggest that the primitive role of *eve* may have been in axial patterning or in the central nervous system. Because cnidarians are unsegmented, and lack any neuron specialization comparable with the insect central nervous system, *eveC* is likely to be involved in axial pattern formation, providing further evidence that this is the 'primitive' function for this class of genes.

We speculate that involvement of *eve* in posterior pattern formation may require positioning at the 5'-end of Hom/Hox clusters. This implies that *eve* homologues should be linked to Hom/Hox clusters in other organisms in which the gene functions in early axial patterning, such as apparently is the case in the grasshopper. Determination of the position of *eve* relative to the Hom cluster in the grasshopper should confirm or disprove this hypothesis.

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- Slack, J. M. W., Holland, P. W. H. & Graham, C. F. *Nature* **361**, 490–492 (1993).
- D'Esposito, M. et al. *Genomics* **10**, 43–50 (1991).
- Boncinelli, E., Simeone, A., Acampora, D. & Mavilio, F. *Trends Genet.* **7**, 329–334 (1991).
- Miles, A. & Miller, D. J. *Proc. R. Soc. B* **248**, 159–161 (1992).
- Schummer, M., Scheurien, I., Schaller, C. & Galliot, B. *EMBO J.* **11**, 1815–1823 (1992).
- Shenk, M. A., Bode, H. R. & Steele, R. E. *Development* **117**, 657–667 (1993).
- Schubert, F. R., Nieselt-Struwe, K. & Gruss, P. *Proc. natn. Acad. Sci. U.S.A.* **90**, 143–147 (1993).
- Patel, N. H., Ball, E. E. & Goodman, C. S. *Nature* **237**, 339–341 (1992).

Yankee doodling

Lewis M. Branscomb

Silicon Samurai: How Japan Conquered the World's IT Industry. By Tom Forester. Blackwell: 1993. Pp. 230. £19.99, \$24.95.

TOM Forester's message is unrelenting: there is no point in resisting inevitable Japanese industrial dominance of information technologies and the markets they support. He has read — and quotes at length — the best doom-sayers, Japan-bashers and US industry critics in Western literature: Clyde Prestowitz, Charles Ferguson, Pat Choate, James Fallows, Karel van Wolferen and so on. For every market sector in which US companies have lost share to the Japanese, his explanation is a bouillabaisse of undifferentiated causes. Sometimes it's the Ministry of International Trade and Industry (MITI)'s prescience and power in directing Japanese industrial strategy; sometimes it's the excellence of Japanese manufacturing; then again it is the blind adherence of the US government to open trade and its failure to adopt a Japanese-style industrial strategy. The picture presented is that of the fourteenth round of the heavyweight battle of Japan versus the West — but is it the West's fatigue and incompetence or Japan's incessant right jabs (and the interference of government officials in its corner) that spell inevitable defeat for the West?

It is perhaps unfair to call this book a selective annotated bibliography of pessimists about the future economic prospects of the United States and Europe. But many of Forester's arguments are set out through quotations from other authors, without reference to or criticism of the original data. His sweeping generalizations are so often unqualified, and at times polemical, that one is suspicious of the data presented in support. When a US company produces a product that is a market failure, as in the case of RCA's video disk, which was technically inferior to its US competitor (MCA and IBM's Discovision), Forester calls it "a great American disaster" that "wrecked this once great US company". Yet he calls Sony's loss of Betamax to the VHS videotape standard "one of [Sony's] few defeats in the consumer electronics market".

This problem is exacerbated by dozens of misleading statements, some of which arise from factual errors, but most of which come from a failure to question the significance of the information presented.

For example, Forester complains throughout the book that "America's failure to capitalize on its inventions is legendary". Many US companies must plead guilty to that charge. But the example he gives (the microwave oven) is irrelevant. It is true that most of the microwave ovens sold today are made in Asia (mostly Korea). But Raytheon Corporation invented its 'Radarange' almost half a century ago. By 1970, half of

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Japanese IT — rising sun or falling star?

Raytheon's revenue (through its subsidiary Amana) came from its 'Radarange', with little Asian competition even though Raytheon granted licences to Japanese manufacturers in the 1960s. Raytheon made billions then; the ovens are a low profit margin commodity now.

Forester also seriously overestimates the contribution of MITI to the development of the Japanese consumer electronics industry, which began with Sony's pioneering success with the transistor radio. In fact, the first transistor radio was developed in 1955 under licence from Western Electric by Dr Ibuka, the founder of Sony; the task took four years and a research and development investment of 12 per cent of annual sales. At the time, the Japanese government controlled the acquisition of licences for foreign

technology, and MITI had refused to allow Sony to license the Western transistor patents because the government was "focusing on rebuilding basic industry". Permission was granted only in 1954. Japanese industrialists are quite ambivalent about MITI's role, although few will say so to Westerners.

More serious, Forester assigns the loss of Silicon Valley's dominance of the microelectronics industry to excessive US entrepreneurship. But the entrepreneurs who left the semiconductor companies to start their own businesses were not interested in the production of large amounts of low-margin DRAMs (dynamic random access memory chips). They were interested in product innovation — new microprocessors and the products built from them. This was a successful strategy, and contributed to today's dominance of microelectronic product innovation and the associated software. The problem was that most of their companies were too small to drive semiconductor process innovations competitively with either the two largest US semiconductor makers, IBM and ATT, or their vertically integrated Japanese competitors. Unfortunately, neither IBM nor ATT sold semiconductors into the merchant market, so their strong position in semiconductor process technology failed to drive the US merchant chip industry. Neither that technology nor the millions of chips they made are adequately reflected in the gloomy statistics portrayed by Forester.

Many of his arguments are inconsistent: Americans fail because they are too individualistic and competitive; yet Japanese succeed because they compete harder. Japan is successful in its exports because it protects a domestic market in which prices are high and products are free from foreign competition. Yet the same policy in Brazil left its information technology industry in the doldrums. Life in the information technology trenches is a lot more complicated than Forester makes out. US industry-bashing and Japan-envy should be explored more objectively and more critically. The starting point is the recognition that no nation coming out of the Second World War with its industry intact, a great reservoir of wartime technology and all its competitors in a shambles, can expect to be still without overseas competitors 40 years later. The shattering of the inevitable US complacency began 20 years ago; the last shards of that complacency have pretty well disappeared.

Forester's underlying assertion is, unfortunately, correct: the large, export-

Takeshi Takahara/SPL

orientated Japanese manufacturing companies, with the full policy support of their government, have grown remarkably. They have shown their willingness to invest heavily in research and engineering and have put to rest any foreign scepticism that their engineers are not clever, inventive and avid readers of the worldwide scientific literature. Their success has no doubt been amplified by tough business tactics and a political effort whose priority is economic success in world markets. US difficulties — including dramatic losses in some markets (machine tools and automobiles especially) — can no doubt be traced more often than not to managerial complacency and excessive attention to short-term returns on capital by the investment community.

The interesting questions are not really addressed. What about the economic slump in Japan? Japanese information technology companies find themselves in difficult financial straits today, much like their US competitors. Their wages and other costs are high; they are saturating the markets of industrialized countries. The really interesting manufacturing competitors, for both Japan and the United States, are emerging in Taiwan, Korea and China. For the first time since the Second World War the big Japanese companies have — like IBM — broken their full employment policy and are cutting back severely on capital investment. Is

their strength seriously affected? Or is their continued investment in research and development once again evidence of their ability to stick the course until the last round? What about the recent resurgence of US industry, despite the economic slow-down? The US share of the semiconductor industry is growing against the Japanese. (The reader will be startled by a figure in the book that shows the sharp reversal in 1989 in the US chip industry's losses to its Japanese competitors, a trend that is still continuing.)

One would hope that the message of a book such as this would be one not of despair but of opportunity. Western companies can learn a lot from the Japanese about how to improve their own performance, and Western governments need to keep their science strong and provide incentives for private investment in technology. My concern is that managers of Western companies and their governments may conclude from Forester's polemic that he and others are crying wolf. They may fail to respond to the Japanese challenge with the focus on technology and innovation that is required to take on such a competent and dedicated set of commercial competitors. □

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Chemical complications

Graham S. Pearson

Veterans at Risk: The Health Effects of Mustard Gas and Lewisite. Edited by Constance M. Pechura and David P. Rall. National Academy Press: 1993. Pp. 448. \$39.95.

DURING the Second World War, US servicemen were used as human subjects in experiments with chemical weapons. Public attention was recently drawn to these 'secret' tests when some of those involved sought compensation from the Department of Veterans Affairs for health problems they said were caused by exposure to mustard gas and lewisite.

In 1991, the department announced guidelines for the handling of the compensation claims, in recognition that the chemical agents may have had some adverse effects on long-term health. In addition, the Institute of Medicine at the US National Academy of Sciences was asked to set up a committee to review the scientific and medical literature on mustard gas and lewisite, to assess the association of exposure with the development of specific diseases, and to identify gaps in the literature and to

recommend ways of filling them.

This book is the committee's report. With more than 100 pages of extensive bibliography and another 100 pages of appendices, it is one of the most comprehensive accounts so far of the short- and long-term effects on health of mustard gas and lewisite. It also offers a historical examination of the use of military volunteers in chemical experiments, reporting on what the soldiers were told before the experiments, how they responded to their exposure to chemical agents and how they were treated afterwards.

Somewhat surprisingly, there is a considerable degree of bias in the report. Of the 17-strong committee, only four are members of the institute and only one is a member of the National Academy of Sciences. There are no representatives of the US Department of Defense or armed services. Throughout, there is a readiness to take the moral high ground and to criticize the armed services for their "secrecy" and "well-ingrained pattern of abuse and neglect", with little attempt to judge the activities of the Second World War by the standards of the time.

Chemical weapons, including mustard

gas and lewisite, were used in the First World War and in regional conflicts between the wars. So they were understandably expected to feature in the Second World War. The committee members seem to forget this, and ignore the fact that there would have been a huge public outcry had there not been any research to improve the protection of the armed forces against chemical weapons. History had already shown that this kind of warfare was more likely to be used against unprotected and poorly protected personnel; the tests were to help in the development of better protective clothing for troops and of ointments that might be effective in reducing blistering.

What of the report's claim that more than 60,000 military personnel were used in the testing programme? The authors urge the Department of Defense to try to identify all these individuals and to notify them "of their exposure and the likely health risks associated with those exposures". Yet, according to the report, only 4,000 of the subjects were exposed to high chemical concentrations, mainly in test chambers and field experiments. The rest took part during military training in the milder "patch tests", in which two drops of mustard were applied to the forearm; one drop was removed immediately using decontaminant, the other two minutes later. The committee members fail to present any evidence of long-term health risks associated with this procedure — so why do they call for the identification of patch-tested individuals? Despite recognizing the psychologically damaging effects of exposure to chemical weapons, the authors nowhere consider the alarm and anxiety that their recommendations would cause thousands of US servicemen and their families.

Overall, the report lacks the balanced judgement and sense of perspective that one would expect from a group of medical and scientific experts. How, for example, was the figure of 60,000 volunteers arrived at? And how did the dosages differ among these subjects? Standards of today are used to judge the exigencies of wartime; an equally strong case for compensation could be made for the effects of passive smoking on nonsmoking military personnel, or for the effects of benzene on those who studied chemistry in the years before this chemical was found to be a carcinogen and banned from school laboratories.

This is not to say that there is no need for follow-up studies of veterans exposed to chemical weapons. But the recommendations of this report will cause needless concern to the many thousands subjected to patch tests. □

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The whole truth?

Brian Charlesworth

Life History Invariants: Some Explorations of Symmetry in Evolutionary Ecology. By Eric L. Charnov. Oxford University Press: 1993. Pp. 167. £25, \$32.50 (hbk); £13.50, \$16.95 (pbk).

THERE has recently been a spate of books on the evolutionary biology of life histories. In contrast to books such as Michael Rose's *Evolutionary Biology of Ageing* and Stephen Stearns's *The Evolution of Life Histories*, which provide wide-ranging reviews, Eric Charnov's book is a research monograph. It focuses on presenting his recent work on life histories, and is aimed at an audience that is already familiar with the basic principles of life-history evolution. I suspect that members of this audience will find it a tough but rewarding read. The writing is terse, and a great deal of mathematical manipulation and data analysis is carried out within a small number of pages.

The intellectual framework of the book is the construction of evolutionary optimization models of life histories, and the comparison of the predictions of these models with data from the literature on life-history variables in a variety of taxa. Simplifying assumptions, such as stationary population size and age-independent mortality of adults, are used to produce the theoretical results. Charnov assumes that the life-history variables under consideration (such as age at maturity) are at evolutionary equilibrium, and can be predicted from the appropriate

trade-off models by maximizing lifetime reproductive success.

This approach will be regarded as annoyingly conservative in some quarters, but it is difficult to see how a broad-brush interpretation of the comparative biology of life histories can be carried out otherwise. The novelty of Charnov's approach lies in the nature of the trade-offs that he invokes. The time-honoured reproductive-effort model, in which present reproduction is assumed to reduce future survival, is discarded. Instead, Charnov ingeniously develops the consequences of trade-offs between growth rate and final body size (for organisms such as fish that grow throughout life) or between growth and reproduction (for species such as mammals that cease to grow around the age at maturity).

Charnov's approach is daring, and remarkably successful in producing quantitative predictions that fit the comparative data. It is a first-rate contribution to life-history evolution. But

there are some questions about whether it is the whole truth, although it surely contains part of the truth. The problem of whether other models of life-history evolution could generate at least some of the patterns revealed by the comparative data is not discussed. Charnov is clearly not interested in the testing of alternative hypotheses, and he pays no attention here to experimental tests of the assumptions of different life-history models. Similarly, I suspect that many evolutionary ecologists will be troubled by such assumptions as that adult body size in mammals is subject to selection solely through the influence of mortality rate and growth rate on optimal age at maturity. The book will undoubtedly play an important role in stimulating thought about where to go next in studies of life-history evolution. □

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Facts at your fingertips

Andrew McMichael

The Leucocyte Antigens FactsBook. By A. Neil Barclay, Marian L. Birkeland, Marion H. Brown, Albertus D. Beyers, Simon J. Davis, Chamorro Somoza and Alan F. Williams. Academic: 1993. Pp. 384. £19.50, \$42 (pbk).

THIS book is a much-needed compendium of information on the leukocyte surface antigens. Alan Williams played a large part in orchestrating the book; he put his last effort into its preparation, finishing only days before his untimely death. This was very much his field; he characterized the first new leukocyte differentiation antigen identified by a monoclonal antibody (CD4) and brought logic to bear on the structures by describing the immunoglobulin superfamily. The avalanche of monoclonal antibodies that followed the first few in the late 1970s was largely sorted out by the series of international workshops that initiated the CD nomenclature system. This book not only catalogues the 78 CD antigens but adds several more that have been sequenced.

The book opens with excellent reviews of the architecture of the cell surface, the protein superfamilies and the chromosomal location of the genes. This part is well illustrated with diagrams, tables and sequence charts. There should be enough information here to place a new sequence in the correct superfamily.

The description of each antigen is admirably concise, with an emphasis on structure, giving the amino-acid sequence in each case. Tissue distribution and func-

tions are summarized, perhaps too briefly, but more can be found in the Leucocyte Typing Workshop volumes (for example, *Leucocyte Typing IV* edited by W. Knapp *et al.*, Oxford University Press, 1992). It is satisfying that so many of the molecules discussed have definable functions, often in adhesion or as receptors; some are paired as receptor-ligands, such as CD2-CD58 and CD5-CD72. The CD3/T-cell receptor complex, major histocompatibility complex molecules and integrins must have presented problems to the authors because of their complexity; they are dealt with concisely and, for those wanting further information, reference is made to other volumes, including R. Piggott's *The Adhesion Molecule FactsBook* (Academic, 1993) for the integrins.

This is an excellent book and a must for all biomedical laboratories with any interest in leukocytes. Of the two copies that I have bought, one has already vanished, a sure sign of an essential volume. The authors must be congratulated on their considerable efforts and it must be hoped that Neil Barclay and colleagues will revise the book or publish regular supplements. The appearance of this volume is very well timed with the fifth Leucocyte Workshop imminent; this will surely yield a new crop of CD antigens, structures and functions to be explored and eventually to go in the next volume. □

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New in paperback

A Complete Guide to the Snakes of Southern Africa by Johan Marais. Blandford, £16.99.

Beyond the Limits: Confronting Global Collapse, Envisioning a Sustainable Future by D. H. Meadows, D. L. Meadows and J. Randers. Chelsea Green, \$14.95. See *Nature* **357**, 371 (1992).

Quantities, Units and Symbols in Physical Chemistry by Ian Mills *et al.* A manual prepared on behalf of the IUPAC. Blackwell Scientific, \$14.95.

Bring Out Your Dead by J. H. Powell. A classic account, first published in 1949, of the great plague of yellow fever in Philadelphia in 1793. University of Pennsylvania Press, \$13.95.

Wheater's Functional Histology: A Text and Colour Atlas by H. G. Burkitt, B. Young and J. W. Heath (3rd edn.) Churchill Livingstone, £27.50.

A Colour Atlas of Human Anatomy by R. M. McMinn, R. T. Hutchings, J. Pegington and P. Abrahams (3rd edn.) Wolfe, £19.95 (also in hbk, £35).

Rises and falls

C. G. A. Harrison

Phanerozoic Sea-Level Changes. By Anthony Hallam. Columbia University Press: 1992. Pp. 266. \$57.50 (hbk); \$28 (pbk).

ONE of the most controversial topics in modern geology is how sea-level changes control sedimentary structures. Changes in sea level can be either eustatic (occurring worldwide) or local, depending on the cause (melting of ice sheets, movements of the ocean floor, sedimentation and so on). Peter Vail and colleagues at Exxon have proposed that seismic reflection records from continental margin sediments and detailed biostratigraphic drilling information give a record of sea-level change. But this idea is not accepted by all geologists. Anthony Hallam, an expert in sea-level studies for more than 30 years, intends to throw some light on the controversy by studying the record of sea-level change as revealed in continental sedimentary deposits.

The book is divided into four main sections. The first, which deals with the techniques of sea-level investigations, is sketchy, and for a detailed understanding of most of the concepts one would have to return to the original literature. An error

has made a nonsense of equation 2.3, and the statement that sedimentation rates are proportional to water depth (p. 42) needs modification — this relationship is roughly true only for water depths less than the depth at which the sediment accumulation rate is greatest.

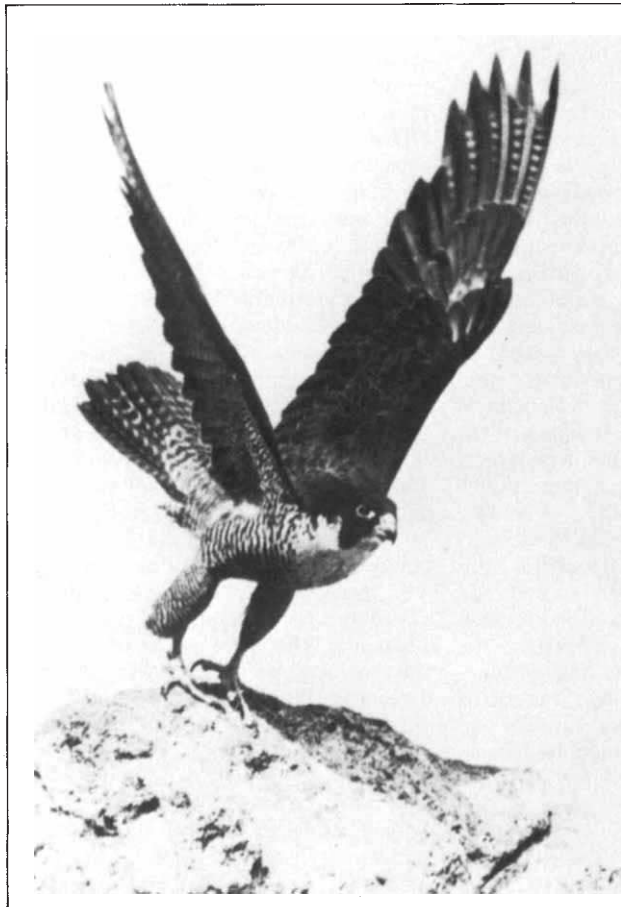
The best part of the book is the second section, which consists of a fairly detailed discussion of the sea-level record in continental deposits throughout the Phanerozoic. It is organized into three chapters, each discussing one era. Hallam compares sea levels as recorded by other scientists with the 'Exxon' curve. Data available to the Exxon group on the Palaeozoic (570–250 million years ago) were sufficiently sketchy that only general trends could be established for this period. But for the more recent Mesozoic and Cenozoic eras the Exxon curve is extremely detailed. On examining the sea-level curves presented for comparison with those from the Exxon group, I was struck not only by the dissimilarity in the timing of the third-order cycles (those whose average duration is about 2 million years) but by the differences in the appearance of the curves. Of course, scientists continue to disagree about how to interpret the curves, but I sometimes sense that Hallam is clutching at straws in identifying correlations between the two sets of curves. Also, there are fewer direct comparisons of sea-level curves than I would have

liked, and on most sea-level curves there are no units for the rises and falls, even though these are often given in the original publications.

In the third part of the book, Hallam discusses the correlation of sea-level change with other phenomena such as isotopic variations, tectonic activity, extinctions and biogeographical changes. The fourth section covers the causes of sea-level change. This is in many ways the weakest of the four sections, but also, to my mind, the most interesting. The only way of producing the sea-level changes of the amount and duration required by the third order Exxon cycles is to invoke the production and melting of large continental ice sheets. As an example, during the Cretaceous there are four sea-level changes greater than 100 m in amplitude, which occur in much less than 1 million years (they appear as instantaneous changes in most representations). Few geologists believe, however, that there were any large continental ice sheets during the Cretaceous. So either the third-order sea-level changes were not eustatic but purely local, possibly produced by tectonic activity, or the changes were smaller or took longer than is suggested by the Exxon curve.

One topic scarcely covered by Hallam is sea-level change over the past century or so, as recorded by tide gauges. There are great variations in these measurements from place to place, variations now interpreted as being due to glacio-isostatic effects. The common signal that remains after accounting for these effects is due to global sea-level change, possibly produced by an anthropogenic factor. Glacio-isostatic effects can tell us much about the viscosity of the mantle, which controls the rate of isostasy, but there are large discrepancies between the various models of glacio-isostasy which need to be resolved. Global sea-level changes could be produced by global warming, either by the warming of surface water to give thermal expansion or by the melting of continental ice sheets. But because the rate of global sea-level change seems to have stayed the same over the past hundred years (at about 1 mm per year), the global warming is unlikely to have an anthropogenic cause, otherwise we would expect there to have been a recent acceleration as the rapidly increasing release of greenhouse gases into the atmosphere takes effect. Teasing apart the changes produced by global change and glacio-isostasy will soon be aided by new space-based geodetic techniques such as very-long-baseline interferometry, satellite laser ranging and the Global Positioning System. □

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WINGS of desire — this photograph comes from the second edition of *The Peregrine Falcon* by Derek Ratcliffe. In a review of the first edition (*Nature* 288, 519; 1980) C. M. White wrote that this detailed book would be "savoured by professional and laymen alike... truly the definitive work on a species that has become a cause célèbre for the environmentally conscious". Species numbers were then in decline owing to the introduction of pesticides; the new edition, however, reports on the reversal of this trend, and in particular on one of the triumphs of wildlife conservation — the full restoration of British and Irish peregrine populations. T&AD Poyser, £25.

Evidence for hotspot-related carbonatite metasomatism in the oceanic upper mantle

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Four peridotite xenoliths from the islands of Savai'i (Western Samoa) and Tubuai (Austral Islands) contain primary olivine of lithospheric origin, and secondary assemblages of clinopyroxene + spinel $\pm$ apatite. The trace-element contents of the secondary minerals show that they formed in equilibrium with carbonate-rich melts. Moreover, isotopic results indicate that these melts were derived from recycled crustal components in the convecting mantle, and place constraints on the origins of these components.

THE presence of volatiles in the Earth's interior, and their role in modifying the mechanical and chemical characteristics of the mantle, is a subject which has received considerable attention in recent years. Volatile-bearing crustal material, in the form of sediments and altered oceanic crust, is clearly identified in the chemistry of convergent margin volcanic rocks. It is uncertain, however, to what extent crustal materials and their associated volatiles are subducted into the convecting mantle. This uncertainty is due, in part, to the limited isotopic variability of hotspot basalts compared with island-arc volcanics, thus rendering the recycling signal somewhat cryptic. Chemical interaction of mantle peridotite with CO₂-rich melts has often been invoked to explain trace element enrichment in alkalic magmas and their included xenoliths of mantle peridotite^{1,3}. Carbonatitic (carbonate-rich) melts can percolate easily through olivine-rich rocks⁴, and signatures of enrichment in rare-earth elements (REE) without concomitant enrichment in high-field-strength elements (Ti, Zr, Hf, Nb, Ta) in mantle-derived peridotites have been interpreted as reflecting chemical interaction with such melts⁵⁻⁸. Recent experiments have established the stability of carbonate minerals in the mantle^{5,9-11}, and show that such minerals could be present in the continental lithospheric mantle and subducting lithosphere, but are unlikely to be stable in the hotter oceanic mantle. All these studies have implied an important, widespread role for carbonatite melts as agents of mantle metasomatism, despite the rarity of these melts at the Earth's surface.

Here we present geochemical results from four oceanic peridotite xenoliths, two each from the islands of Savai'i (Western Samoa, Samoa hotspot) and Tubuai (Austral Islands, Macdonald hotspot). These peridotites contain primary mineralogies of olivine $\pm$ orthopyroxene of oceanic lithosphere origin, and secondary assemblages of clinopyroxene + spinel $\pm$ apatite $\pm$ glass which were derived from interaction of carbonatitic melts with the lithospheric protoliths. Isotopic data on clinopyroxenes and glass inclusions demonstrate that these melts were derived from mantle sources with extreme isotopic compositions, similar to those which gave rise to the basaltic volcanism at these hotspots, and show that these sources do not reside in the oceanic lithosphere. These data provide strong support for crustal and volatile recycling into the convecting mantle, particularly the source regions of the Samoa and Macdonald hotspots.

Petrography and major-element chemistry

Mineral compositions and point-counted phase abundances are shown in Tables 1 and 2, respectively. 90SAV 1-1 is a 4 cm $\times$ 2 cm harzburgite from Savai'i, Western Samoa consisting of primary

olivine (ol1, 1–10 mm, Fo₉₁) and orthopyroxene (opx, 0.3–3 mm), with patches of secondary clinopyroxene (cpx2, 0.1–2 mm), chromite (sp, 0.01–0.1 mm), trace F-rich apatite (ap, 0.1–1 mm) and glass (gl). Primary olivine and opx define a mildly porphyroclastic texture, with 0.5–2 mm patches of cpx + sp $\pm$ opx $\pm$ ap $\pm$ gl distributed in the interstices in a heterogeneous fashion. These patches are in various stages of reaction, ranging from glass-free cpx $\pm$ opx + sp symplectites with adjacent large apatite grains in textural equilibrium (Fig. 1a, b), to patches with relict cpx + sp reacting to form ol + gl. No relict opx is present in glass-bearing patches. Olivine neoblasts adjacent to glass-free patches (secondary olivine, ol2, 0.01–0.2 mm) have higher Mg# (Mg number, defined as 100 Mg/(Mg + Fe)) and higher CaO levels than primary olivine; this is also true of olivine in glass pockets. Clinopyroxene is not a phenocryst phase in glass pockets except where a vein of host basalt intrudes; in this case, the secondary clinopyroxene cpx2, has a very different composition, similar to microphenocrysts in the host basalt.

90SAV 1-28 is a 2 cm $\times$ 2 cm harzburgite from Savai'i containing primary olivine (Fo₉₁, 1–20 mm) and opx (0.3–5 mm), with interstitial cpx (0.05–2 mm) and sp (0.01–0.1 mm) forming cpx + sp symplectites (0.1–1 mm). Clinopyroxene also forms thin intergranular rims around olivine and opx. small ol + sp symplectites (0.01–0.2 mm) are rare. No glass or apatite is present in this xenolith. The texture is protogranular with a well annealed appearance.

TBA 1-9 is a 2 cm $\times$ 2 cm wehrlite from Tubuai (Austral Islands) consisting of primary olivine (Fo₉₀, 0.1–1 mm) and minor opx (0.05–0.3 mm) forming a mildly porphyroclastic texture. Approximately 40% by volume of the sample consists of cpx + gl + sp $\pm$ ap melt pockets, with oriented cpx, relict chromite, and often euhedral F-rich apatites as phenocrysts crystallizing from a silica-alumina-alkali rich glass (Fig. 1d). The cpx has an unusual Al-poor, Cr-rich composition with a high Mg# (Table 1), and is very similar to endiopsides described by Daturia *et al.*⁷. Olivine is not a phenocryst phase in the melt pockets. The morphology of these melt pockets varies, but often they have straight boundaries or rectangular habits resembling pseudomorphs after a single phase, and the glass rarely intrudes into the ol + opx matrix. Reconstructed compositions of these melt pockets (Table 1) resembles strongly a subcalcic pyroxene composition ($\pm$ apatite). Clinopyroxene is not present outside these melt pockets.

TBA 4-11 is a 1 cm $\times$ 2 cm wehrlitic dunite consisting of large primary olivine (Fo₈₆₋₈₈, 1–10 mm) enclosed in a finer grained matrix of ol2 (Fo₈₄₋₉₀, 0.1–1 mm) with patches of cpx (0.05–

0.2 mm), chromite (0.01–0.1 mm) and F-rich apatite (0.05–2 mm) (Fig. 1c). The texture and olivine compositions suggest a largely cumulate origin for TBA 4-11, but olivines with compositions up to $\text{Fo}_{90.3}$ occur as well; the olivines with the highest Mg# are associated with $\text{cpx} \pm \text{sp}$ patches and have high CaO levels. Silica-rich glass usually makes up 5–10 vol% of these patches.

Exsolution features are absent in these xenoliths, except for very fine pyroxene lamellae in 90SAV 1-28. Primary olivines in all samples contain abundant fluid and melt inclusions along annealed fractures. The composition of olivine-hosted melt inclusions is generally similar to that of interstitial glasses. No hydrous phases such as amphibole or phlogopite have been observed in these samples, and other peridotites from both localities are notable for the complete absence of hydrous phases. Patches of $\text{cpx} + \text{sp} \pm \text{ap} \pm \text{gl}$ are distributed heterogeneously in all samples. When they coexist, apatite is reacting with glass (except TBA 1-9), but isolated apatite grains are unreacted. These apatites contain abundant inclusions, and resemble apatites described by Yaxley *et al.*⁶ in xenoliths from S. E. Australia. The presence of silica-alumina-alkali rich glasses in the cpx patches are tertiary features, probably produced by volatile-aided incongruent dissolution of pyroxenes during heating and decompression of the xenoliths. Phases associated with the $\text{cpx} + \text{sp} \pm \text{ap} \pm \text{gl}$ patches have invariably higher Mg# (and higher Cr# (defined as $\text{Cr}/(\text{Cr} + \text{Al} + \text{Fe}^{3+})$ for spinels) than the primary mineralogy. This lack of an Fe-Ti enrichment trend

indicates that the metasomatic agent was not an Fe-bearing silicate melt⁸.

Signature of carbonatite metasomatism

Trace-element analyses of cpx, opx, apatite and glass were determined by ion microprobe techniques (except U, Th and Pb by isotope dilution); the results are given in Table 1 and displayed graphically in Fig. 2. Clinopyroxenes from these samples are characterized by very high concentrations of the light-rare-earth elements (LREE) La, Ce and Nd, high concentrations of Sr, U, Th and Pb, but remarkably low concentrations of Ti. The level of Rb in cpx and opx from all xenoliths is <0.1 parts per million (p.p.m.). Analyses of opx show variable enrichment in REE and depletion in Ti; calculated cpx/opx partition coefficients are generally high, and probably indicate disequilibrium. Apatite is characterized by much higher concentrations of REE, Sr and Ba relative to coexisting cpx.

The depletion of Ti, Zr and Nb in cpx cannot be due to partitioning into a coexisting Ti-bearing phase because no such phase was observed during a detailed search using back-scattered electron imaging. No hydrous phases such as amphibole or phlogopite, which may contain substantial quantities of these elements, are present in these xenoliths. The trace-element composition of the bulk-rock peridotites is shown in Fig. 2e, calculated from the compositions and abundances of olivine, opx, cpx, spinel, apatite and glass. These bulk rock patterns are also depleted in Ti, Zr, and Nb (as well as Sr) relative to the REE,

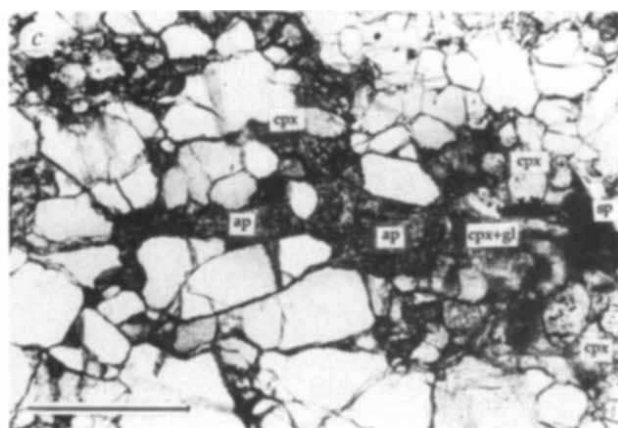
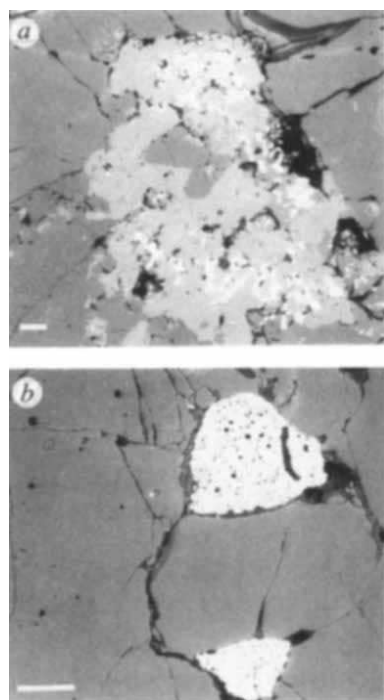


FIG. 1 a, Backscattered SEM image of clinopyroxene + chromite aggregate around relict orthopyroxene, surrounded by olivine (dark grey), 90SAV 1-1. Clinopyroxene (cpx) is light grey, chromite is white, and relict orthopyroxene (opx) in the centre of the patch is dark grey. This assemblage provides evidence for the carbonatitic reaction $\text{opx} + \text{CaCO}_3 \rightarrow \text{cpx} + \text{CO}_2$ described in ref. 11. Scale bar on the left is 100 μm. b, Backscattered SEM image of apatite crystals (white) in textural equilibrium with surrounding olivine (grey), 90SAV 1-1. Scale bar on the left is 100 μm. c, Aggregate of large apatite (ap) crystals (dark grey, cloudy) and clinopyroxene surrounded by high Mg#, high CaO olivines (white), TBA 4-11. Apatite + olivine + clinopyroxene + chromite (sp) area contains about 10% silica-rich glass (gl). Transmitted light scale bar is 1 mm. d, Melt pocket of euhedral Al-poor, Cr-rich diopside,

with relict chromite (sp, dark, upper right) in silica-rich glass, surrounded by olivine, TBA 1-9. Glass also contains euhedral apatite not visible in this photo. This texture probably represents breakdown of an original precursor pyroxene ($\pm \text{apatite} \pm \text{spinel}$). Transmitted light scale bar is 1 mm.

reflecting similar depletions in the constituent phases. These patterns are similar to those found in continental xenoliths that have been affected by carbonatite metasomatism, such as the peridotites and wehrlites described by Yaxley *et al.*⁶ and Daturia *et al.*⁷, but have higher concentrations of all elements (except Ti and Nb) than dunites reported by Rudnick *et al.*⁸.

The trace-element compositions of these xenoliths reflect the

melt (or melts) responsible for trace-element enrichment in these samples. The trace-element composition of this melt can be estimated by calculating the composition of a hypothetical melt which would be in equilibrium with cpx. Partition coefficients between cpx and carbonatite melts have been determined recently for several elements^{12,13}, and with the exception of Ti¹³, these values are all similar to cpx/basaltic melt partition

TABLE 1 Major and trace element and isotope compositions of xenolith phase

	Clinopyroxene analyses						Orthopyroxene analyses				D (cpx/melt)		
	90SAV 1-1 cpx1	90SAV 1-1 cpx2	90SAV 1-28 cpx1	TBA 1-9 cpx2	TBA 1-9 melt pocket	TBA 4-11 cpx1	90SAV 1-1 opx	90SAV 1-28 opx	TBA 1-9 opx				
SiO ₂	54.69	49.13	53.73	54.90	55.84	49.66	57.52	56.50	55.40				
TiO ₂	0.02	2.14	0.17	0.11	0.25	2.28	0.01	0.02	0.01				
Al ₂ O ₃	1.40	6.46	2.09	0.58	6.61	4.34	1.25	2.66	2.97				
Cr ₂ O ₃	1.59	1.44	2.03	3.21	2.26	1.09	0.48	0.74	0.76				
CaO	19.89	22.10	22.52	21.97	16.10	23.53	0.96	0.73	0.72				
MgO	17.74	14.66	16.79	16.31	11.68	14.71	34.30	34.33	33.55				
FeO	2.65	3.35	2.45	1.70	1.76	4.06	5.28	5.45	6.04				
MnO	0.06	0.08	0.10	0.11	0.08	0.07	0.08	0.11	0.12				
Na ₂ O	2.31	1.54	1.02	1.73	3.11	0.79	0.10	0.07	0.00				
K ₂ O	0.00	0.00	0.00	0.00	0.14	0.00	—	—	—				
Sum	100.35	100.90	100.90	100.62	97.83	100.53	99.98	100.61	99.57				
Mg #	0.923	0.886	0.924	0.945	0.922	0.866	0.921	0.918	0.908				
Ba	0.92	0.10	0.78	1.87		0.57				0.0006			
Th (ID)	2.79					0.693				0.013			
U (ID)	0.788					0.225				0.006			
Pb (ID)	2.44					0.358				0.010			
Nb	0.54	0.10	0.31	1.30		0.87				0.0075			
La	102	11.7	75.2	21.8		67.7	0.90	0.13	0.22	0.060			
Ce	190	36.4	232	76.4		189	2.06	0.19	0.45	0.10			
Sr	541	99.3	353	838		460	18.3	0.22	0.53	0.13			
Nd	58.1	26.4	125.0	61.2		103	0.99	0.33	0.26	0.20			
Zr	71.6	50.7	75.4	13.4		139	3.30	2.2	2.6	0.17			
Sm	8.38	7.44	25.10	17.4		23.8	0.28	0.09	0.11	0.30			
Eu	1.44	2.32	5.93	2.79		7.40	0.10	0.04	0.04	0.38			
Ti	27.7	10950	687.0	185		314	15.3	7.4	22.4	1.2			
Dy	2.63	4.55	9.93	11.2		15.1	0.44	0.23	0.19	0.44			
Er	1.26	3.46	4.51	5.92		7.94	0.32	0.18	0.13	0.45			
Yb	0.55	2.24	3.78	4.56		6.56	0.40	0.28	0.17	0.45			
⁸⁷ Sr/ ⁸⁶ Sr	0.712838		0.710969	0.702796		0.702755							
¹⁴³ Nd/ ¹⁴⁴ Nd	0.512516		0.512467	0.512902		0.512942							
²⁰⁶ Pb/ ²⁰⁴ Pb	18.845		18.877	21.313		20.971							
²⁰⁷ Pb/ ²⁰⁴ Pb	15.660		15.642	15.751		15.748							
²⁰⁸ Pb/ ²⁰⁴ Pb	39.806		39.720	40.598		40.228							
	Glass analyses			Apatite analyses			Olivines						
	90SAV 1-1	TBA 1-9	TBA 4-11	90SAV 1-1	TBA 1-9	TBA 4-11	90SAV ol1	90SAV 1-1 ol2	90SAV 1-28	TBA 1-9	TBA 4-11 ol1	TBA 4-11 ol2	
SiO ₂	69.24	60.03	55.22	0.27	0.31	0.38	SiO ₂	40.67	40.77	40.77	40.51	40.18	40.39
TiO ₂	0.20	0.61	0.14				TiO ₂	0.00	0.00	0.00	0.00	0.06	0.04
Al ₂ O ₃	16.41	21.40	24.78	0.06	0.09	0.00	Al ₂ O ₃	0.04	0.00	0.04	0.03	0.07	0.01
Cr ₂ O ₃	0.05	0.05	0.01				Cr ₂ O ₃	0.01	0.02	0.12	0.04	0.06	0.10
CaO	1.57	0.71	4.45	52.16	51.87	53.98	CaO	0.04	0.39	0.12	0.03	0.22	0.38
MgO	1.22	0.90	0.81	0.43	0.43	0.34	MgO	49.77	50.47	49.76	49.10	46.06	49.32
FeO	1.77	1.96	0.56	0.19	0.22	0.19	FeO	8.66	8.16	8.99	9.54	12.79	9.50
MnO	0.05	0.01	0.01				MnO	0.10	0.09	0.10	0.17	0.29	0.15
P ₂ O ₅	0.11	0.26	0.07	40.85	39.90	40.33	NiO	0.35	0.37	0.35	0.30	0.24	0.29
Na ₂ O	4.05	6.54	6.01				Sum	99.66	100.25	100.26	99.73	99.96	100.16
K ₂ O	4.13	0.47	4.46				Mg #	0.911	0.917	0.908	0.902	0.865	0.902
F				4.82	5.51	4.40	¹⁸⁷ Os/ ¹⁸⁶ Os	1.0717				1.0725	
Cl				0.30	0.30	0.23							
Sum	98.80	92.92	96.51	99.09	98.62	99.85							
Mg #	0.551	0.451	0.722										
	Glass analyses			Apatite analyses			Olivines						
	90SAV 1-1	TBA 1-9	TBA 4-11	90SAV 1-1	TBA 1-9	TBA 4-11	90SAV ol1	90SAV 1-1 ol2	90SAV 1-28	TBA 1-9	TBA 4-11 ol1	TBA 4-11 ol2	
Ba	540	790	214	538	845	1040							
Nb	200	506	347	0.1	0.1	0.1							
La	190	514	598	1400	2006	2940							
Ce	333	595	660	3420	5552	7000							
Sr	860	2020	761	7050	9840	12300	SiO ₂	0.01	0.06	0.00	0.00		
Nd	151	151	172	1240	3272	2280	TiO ₂	0.15	0.17	0.01	0.01		
Zr	419	266	592	6.14	4	7.74	Al ₂ O ₃	15.40	33.25	6.80	15.20		
Sm	30.0	17.2	19.9	155	638	263	Cr ₂ O ₃	53.50	32.91	64.40	53.80		
Eu	7.60	2.65	6.29	49	197	81	CaO	0.12	0.10	0.01	0.00		
Ti	1340	2580	880	9.17	6.11	11.25	MgO	15.30	15.95	15.14	15.30		
Dy	17.0	11.8	12.8	72	234	119	FeO	11.20	13.10	10.05	11.20		
Er	11.6	7.20	7.68	32	92	62	Fe ₂ O ₃	4.05	4.93	2.93	4.10		
Yb	6.50	6.22	6.96	20	51	45	MnO	0.27	0.22	0.45	0.29		
							NiO	0.11	0.27	0.20	0.07		
⁸⁷ Sr/ ⁸⁶ Sr	0.712344		0.702781				Sum	99.95	100.73	99.98	99.96		
¹⁴³ Nd/ ¹⁴⁴ Nd	0.512422		0.512916				Mg #	0.709	0.685	0.729	0.709		
²⁰⁶ Pb/ ²⁰⁴ Pb	18.849		20.966				Cr #	0.700	0.399	0.864	0.704		
²⁰⁷ Pb/ ²⁰⁴ Pb	15.669		15.748										
²⁰⁸ Pb/ ²⁰⁴ Pb	39.845		40.275										

Major-element data (weight %), trace-element data (parts per million) and isotope ratios for minerals from Savai'i and Tubuai peridotite xenoliths. Major elements were determined by electron microprobe. All elements (except U, Th and Pb) were determined *in situ* by ion microprobe, based on calibrations using natural mineral standards. Ba and Nb in clinopyroxene were standardized on basalt glass. U, Th and Pb were determined by isotope dilution on separated clinopyroxene. Analytical uncertainty is estimated at 3% for U, Th and Pb, 20–30% for Ba, Nb and Eu, and 15% for all other elements. Analytical uncertainty is 0.004% for Sr and Nd isotope data, 0.1% per a.m.u. for Pb isotope data. Uncertainty is 0.3% for Os isotope data on olivine separates. Partition coefficients are from refs 13–15.

coefficients (within a factor of two). Values of these latter partition coefficients have recently been determined simultaneously in individual experiments^{14,15} for all the elements in Table 1. (This experimental strategy has the advantage of minimizing errors in the relative values of partition-coefficients.) These values (except the Ti partition coefficient, which is taken from ref. 13) are used to calculate the putative equilibrium melts for cpx from these xenoliths and the results are displayed in Fig. 3. As expected, these putative equilibrium melts are also characterized by depletion of Ti, Zr and Nb relative to adjacent REE. These calculated trace-element patterns are compared in Fig. 3 with the trace-element data for carbonatites reported by Nelson *et al.*¹⁶ and representative basalts from Samoa and Tubuai¹⁷⁻¹⁹. The basalts lack the high concentrations of LREE and depletion of Ti, Zr and Nb needed to generate the observed cpx compositions. However, the match between the carbonatites and the melts in equilibrium with the xenolith clinopyroxenes is nearly perfect for all the elements investigated. This demonstrates that the secondary cpx + sp ± ap assemblages were equilibrated with a melt that was very similar to carbonatite in its trace-element composition. The only notable difference is that the calculated equilibrium melts have high La/Nb ratios relative to most continental carbonatites, possibly reflecting an origin in the oceanic mantle or open-system metasomatism.

Trace-element patterns of the silica-rich glasses also compare very well with carbonatites (Fig. 2). The characteristic depletions

of Ti and Zr observed in cpx also occur in the silica-rich glasses in these xenoliths, but the glasses do not show the distinctive depletion of Nb that occurs in cpx. These features are similar to those found for the glass pockets described by Ionov *et al.*²⁰. Isotopic compositions of glass inclusions from 90SAV 1-1 and TBA 4-11 (which are similar in major-element composition to the interstitial glasses) are distinct from those of the host basalts enclosing the xenoliths. A suggested origin of these glasses from decompression melting of amphibole or phlogopite is inconsistent with their silica-saturated compositions, as well as the complete absence of such phases in these xenoliths. This observation implies anhydrous conditions for metasomatism. The trace-element patterns of cpx and glass (Fig. 2) are roughly consistent with predicted partitioning relationships between clinopyroxene and silica-rich melts²¹, suggesting that these glasses probably formed as tertiary features by partial melting of secondary cpx + sp ± ap assemblages. This is supported by the observation that the trace-element patterns of the glasses resemble mixtures between cpx and apatite compositions (Fig. 2). However, the isotopic differences between glass inclusions and cpx in 90SAV 1-1 argues for a more complex origin for these glasses. Similar silica-rich glasses have been observed to coexist with carbonatite melt and CO₂ vapour within single olivine-hosted inclusions in peridotite xenoliths from Kerguelen Island, with trapping pressures in excess of 1.2 GPa²². We speculate that the glasses in the Savai'i and Tubuai xenoliths may have formed through

FIG. 2 Trace-element patterns for xenolith minerals and glasses, normalized to C1 chondrite values of Anders and Grevesse³⁷ (except Pb=0.060 p.p.m.). All phases are characterized by negative anomalies of Ti and Zr relative to the rare earth elements (REE). e Shows the trace-element composition of the bulk peridotites calculated from the compositions and modal abundances of olivine, orthopyroxene, clinopyroxene, spinel, glass and apatite. The bulk-rock patterns are all characterized by depletion of Ti, Zr, Sr and Nb relative to the REE, consistent with carbonatite metasomatism.

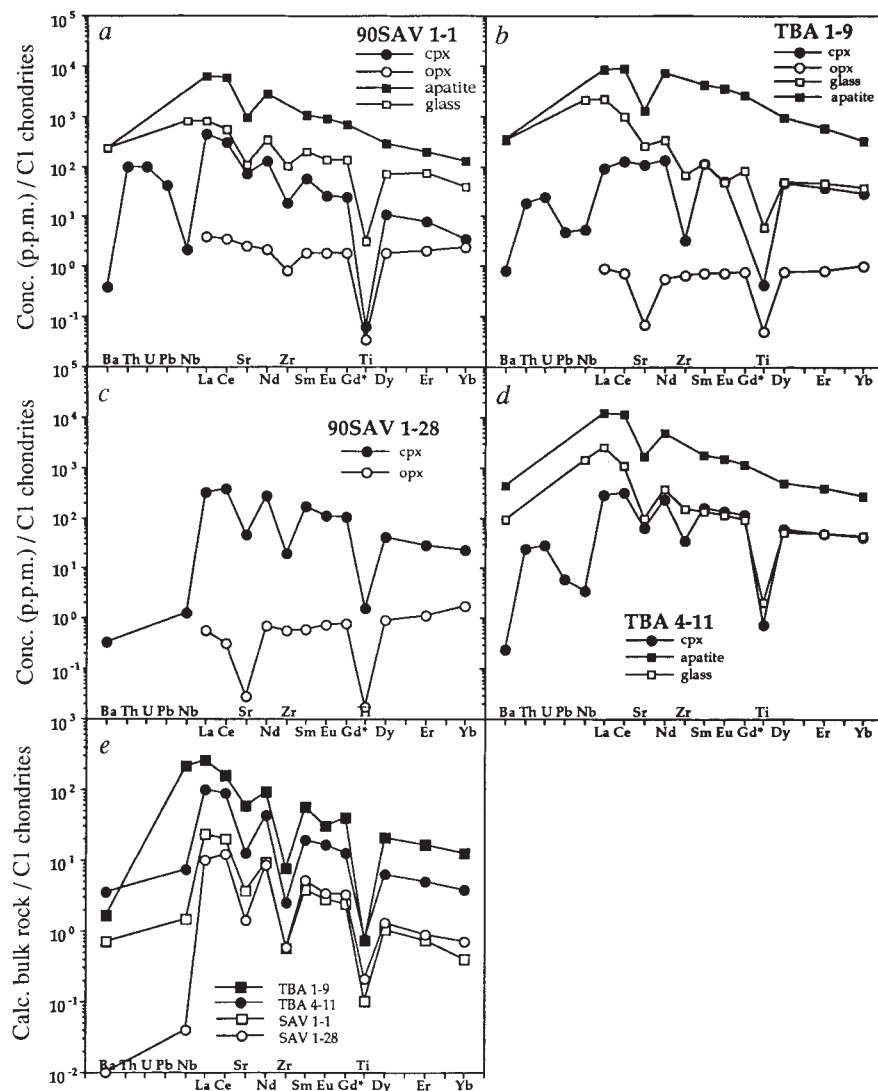


TABLE 2 Whole-rock xenolith compositions

Phases	90SAV 1-1	90SAV 1-28	TBA 1-9	TBA 4-11
Olivine	0.814	0.678	0.529	0.931
Orthopyroxene	0.15	0.279	0.05	0
Clinopyroxene	0.021	0.031	0.28	0.05
Spinel	0.01	0.012	0.015	0.005
Glass	0.002	0	0.12	0.006
Apatite	0.003	0	0.004	0.008
Calculated bulk rocks				
	90SAV 1-1	90SAV 1-28	TBA 1-9	TBA 4-11
SiO ₂	43.00	45.07	45.48	40.17
TiO ₂	0.01	0.02	0.09	0.17
Al ₂ O ₃	0.48	1.34	2.56	0.50
Cr ₂ O ₃	0.79	0.86	2.19	0.45
CaO	0.71	0.98	6.43	1.72
MgO	46.23	44.08	32.60	43.72
FeO	8.04	7.89	6.21	12.19
MnO	0.10	0.10	0.14	0.27
P ₂ O ₅	0.09	0.00	0.14	0.24
Na ₂ O	0.07	0.05	1.13	0.07
K ₂ O	0.01	0.00	0.05	0.02
Sum	99.53	100.40	97.01	99.50
Mg #	0.911	0.909	0.903	0.865
Ba	2.71	0.02	98.0	9.64
Nb	0.34	0.01	50.2	1.75
La	5.52	2.33	62.5	23.5
Ce	12.0	7.19	96.2	53.6
Sr	28.2	10.9	46	98.6
Nd	4.18	3.88	41.6	19.3
Zr	2.20	2.34	29.9	9.91
Sm	0.56	0.78	8.43	2.82
Eu	0.15	0.18	1.62	0.87
Ti	45.1	90.6	321	328
Dy	0.24	0.31	4.98	1.51
Er	0.12	0.14	2.64	0.80
Yb	0.07	0.12	2.04	0.63

Point-counted phase abundances ($n > 3,000$ points), and reconstructed major- and trace-element compositions of bulk rocks. Compositions (major and trace elements) of bulk rocks were calculated from phase compositions and phase abundances.

reaction of either solid carbonate or CO₂ with secondary cpx + sp ± opx ± ap during heating of these xenoliths in the mantle, further modified by decompression during transport to the surface.

Xenolith-basalt relationships

Isotopic data are given in Table 1 for separates of clinopyroxene and olivine-hosted inclusions (Sr, Nd, Pb) and porphyroclastic olivine (Os). The Pb isotope results for cpx and glass inclusions from Samoan xenoliths have high ²⁰⁷Pb/²⁰⁴Pb ratios that are similar to the highest values reported for Samoan basalts, but the ²⁰⁸Pb/²⁰⁴Pb ratio is higher than that in any Samoan basalt^{23,24}. The ⁸⁷Sr/⁸⁶Sr ratio is much higher, and the ¹⁴³Nd/¹⁴⁴Nd ratio is much lower, than the extreme values for Samoan basalts (Fig. 4), and these analyses lie on a curved extension of the EMII mantle composition²⁵ (see Fig. 4 legend). Based on the age of the oceanic crust beneath Savai'i (~100 Myr) and measured Rb/Sr, Sm/Nd and U, Th/Pb ratios, this isotope signature could not have been created in the oceanic lithosphere after its formation at the East Pacific Rise. This point is emphasized by the different isotopic composition of the cpx from the Tubuai xenoliths, despite the trace-element similarities. The Sr–Nd–Pb isotope signature of the Tubuai xenoliths are similar (but not identical) to basalts from Tubuai, which lie at the low ⁸⁷Sr/⁸⁶Sr–high ²⁰⁶Pb/²⁰⁴Pb end of the Macdonald hotspot array (Fig. 4b), characteristic of the HIMU mantle composition²⁵ (see Fig. 4 legend). The existence of carbonate in the mantle beneath Tubuai has been suggested previously³⁸. Our trace-element results indicate that the carbonatitic imprint dominates the Sr–Nd–Pb budgets of these xenoliths, and thus their Sr–Nd–Pb

isotopic signatures. The Os isotopic compositions of olivine separates, however, are within the range expected for oceanic lithosphere^{26,27}, and in particular the ¹⁸⁷Os/¹⁸⁶Os ratios for TBA 4-11 are well below the values reported for Tubuai basalts³⁰. These results suggest that the budget of compatible elements in these xenoliths, including Os, were largely unaffected by the carbonatitic metasomatism, and indicate that olivine porphyroclasts in these xenoliths are largely primary. This is consistent with the generally low concentrations of compatible elements in carbonatites¹⁶. These observations suggest that the carbonatitic melts responsible for metasomatism were either generated directly by partial melting of isotopically extreme carbonate-bearing sources in the mantle plumes beneath these islands²⁸, or were evolved in the lithospheric mantle from extremely CO₂-rich melts derived from the same sources²⁹.

Several lines of evidence indicate that these xenoliths, derived from the oceanic lithosphere do not represent sources for basaltic volcanism at the islands of Samoa and Tubuai. Xenoliths of this type are quite rare, representing only two of 60 from Savai'i, and two of 20 from Tubuai; in addition these trace-element characteristics have not been reported from any other oceanic islands. Major-element compositions, except for the wehrlite TBA 1-9, are quite refractory, and could not yield substantial amounts of basaltic melt. Furthermore, the isotopic data indicate that the trace-element enrichments are young features, related to melt migration processes during the periods of volcanic activity at the islands of Savai'i and Tubuai. The combined Sr, Nd, Pb and Os isotopic signatures of these xenoliths do not match those of basalts at these islands, due to a metasomatic origin for

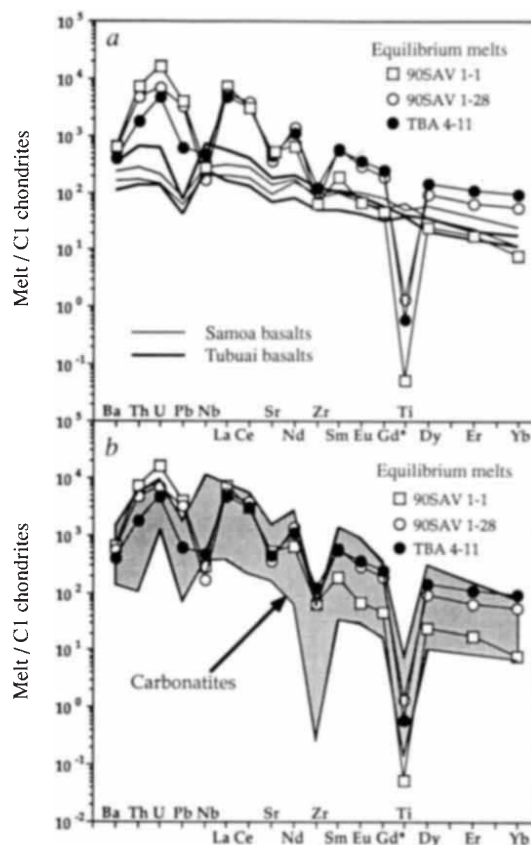


FIG. 3 Calculated trace-element patterns for putative melts in equilibrium with clinopyroxene from Savai'i xenoliths (open symbols) and Tubuai xenolith TBA 4-11 (closed circles) compared with basalts from Samoa and Tubuai (a) and carbonatites from various localities (b). Melt compositions were calculated by dividing clinopyroxene concentrations by clinopyroxene/basaltic melt partition coefficients in Table 1. Samoa and Tubuai basalt data are from refs 17–19. Shaded field encloses trace-element data for carbonatites reported by Nelson *et al.*¹⁶.

the Sr, Nd and Pb isotopes, and a lithospheric origin for the Os isotopes in the peridotites. These xenoliths provide abundant evidence against lithospheric sources for endmember isotopic compositions which contribute to much of the isotopic variability in oceanic island basalts, in particular those from the Samoa and Macdonald island chains.

Isotopic evidence for recycled crust

These results indicate that the lithospheric mantle beneath the islands of Savai'i and Tubuai were metasomatized by carbonatitic melts, which in turn were derived from the same sources that gave rise to basaltic volcanism at these islands. These sources are located in the convecting mantle and were probably delivered in mantle plumes. At both islands, the Sr–Nd–Pb isotopic compositions of the xenoliths indicate an association of the carbonatitic component with the most extreme endmember compositions found in these island chains. The xenolith data provide important constraints on the origins of the EMII and HIMU mantle compositions.

The homogeneity of the Sr–Nd–Pb isotopic compositions of extreme HIMU basalts from Tubuai and Mangaia indicates that the HIMU signature (observed in both these basalts and the Tubuai xenoliths) was a dominant component in the Macdonald plume during the period of Tubuai volcanism. As a general

feature, the scatter of Sr–Nd–Pb isotopic data for ocean island basalts decreases substantially toward the HIMU endmember. This is consistent with the hypothesis that HIMU represents recycled oceanic crust in the convecting mantle^{25,30–32}, because the Sr–Nd–Pb budget of a mixture of oceanic crust and peridotite would be strongly controlled by the MORB crustal component, and would be expected to be only as heterogeneous typical MORB crust.

The origin of the high-⁸⁷Sr/⁸⁶Sr trend in the Samoa basalt data has been used as evidence for recycled sediments in the convecting mantle²³. The Sr–Nd–Pb isotopic data for the Savai'i xenoliths are similar to many modern sediments^{33–35}, and are reminiscent of isotopic trends in island arc volcanics³³. It is possible that this similarity indicates a rather short residence time for the sedimentary component in the convecting mantle. The similar Pb isotopic compositions of the Savai'i xenoliths and the Samoan basalts indicates that the Pb in most Samoan lavas is largely dominated by the Pb derived from the sedimentary component, but the basalt Sr and Nd budget is only partially sedimentary. This is consistent with the concentrations of Pb, Sr and Nd in most oceanic sediments compared to mantle peridotite^{33–35}. The observation that the Sr–Nd–Pb isotopic signatures of the Samoan xenoliths are outside the range of Samoan basalts indicates that the extreme EMII component is probably a volumetrically minor constituent in Samoan lavas, and by inference in the Samoan plume. It also indicates that melt generation and segregation processes acted to dilute this signature with melts of less extreme isotopic compositions before the basalts were erupted. However, this extreme EMII component is a major component of the carbonatitic melt. This is consistent with the observation that isotopic data for EMII ocean island basalts do not converge strongly towards a unique high-⁸⁷Sr/⁸⁶Sr composition, but tend to scatter. It is significant that the isotopic compositions of the Savai'i xenoliths also do not converge, nor do they define a single mixing array between sedimentary and mantle components. This either indicates heterogeneity within the sedimentary component, or that the actual isotopic composition of the sedimentary component may be even more extreme than 90SAV 1-1. It thus appears that both of the Samoan xenoliths contain a melt fraction trapped at depth which is observed only in a diluted form in Samoan basalts.

Clear evidence for carbonatitic metasomatism is present in these peridotite xenoliths, hosted in ocean island basalt, from Samoa and Tubuai. It is possible that detailed investigation of trace-element signatures will uncover similar xenoliths at other oceanic islands, and may reveal evidence of melt fractions trapped within the lithosphere which are not observed at the surface. This study demonstrates the volatile (CO₂)-rich nature of the EMII and HIMU mantle components, consistent with their origins as recycled sediments and oceanic crust, respectively, and reinforces other observations of crustal recycling into the convecting mantle³⁶. The association of these recycled isotopic signatures with carbonatite metasomatism indicates that some mechanism must exist to transport CO₂, and probably other volatiles as well, through subduction zones and into the convecting mantle. □

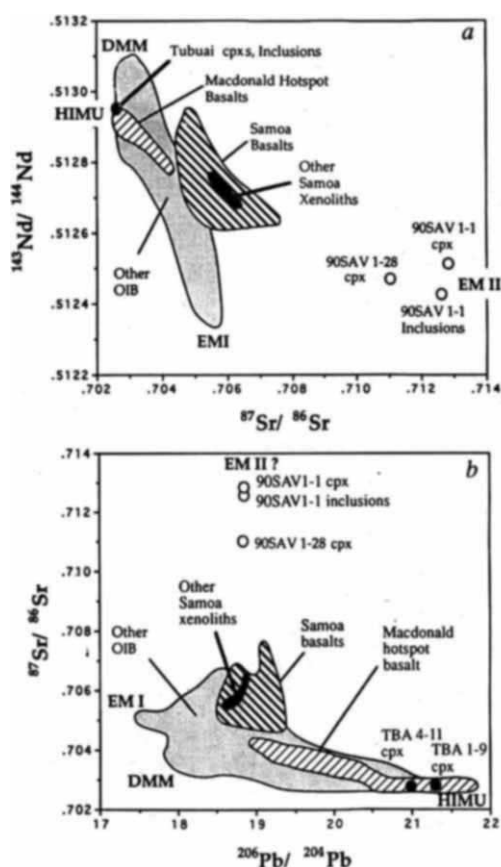


FIG. 4 a, Nd–Sr and b, Sr–Pb isotope diagrams comparing isotope data for xenolith clinopyroxene separates and glass inclusions with data for basalts from Samoa^{17,23,24} and Tubuai^{19,27} and other oceanic basalts²⁵. Isotopic mantle endmembers are only generally defined²⁵, and represent the depleted MORB source mantle (DMM), a high U/Pb component (HIMU), and two enriched mantle components (EMI and EMII). The position of the EMII endmember is uncertain. As the Samoa xenolith data do not define a linear mixing array, this implies that 'pure' EMII is either heterogeneous, or has higher ⁸⁷Sr/⁸⁶Sr and lower ¹⁴³Nd/¹⁴⁴Nd ratios than indicated. The Pb isotope composition of EMII, however, appears to be very well constrained by the Samoan xenolith data.

Received 29 March; accepted 12 August 1993.

1. Menzies, M. A. & Wäss, S. Y. *Earth planet. Sci. Lett.* **65**, 287–320 (1983).
2. O'Reilly, S. Y. & Griffin, W. L. *Geochim. cosmochim. Acta* **52**, 433–447 (1988).
3. Dupuy, C., Liotard, J. M. & Dostal, J. *Geochim. cosmochim. Acta* **56**, 2417–2423 (1992).
4. Hunter, R. H. & McKenzie, D. *Earth planet. Sci. Lett.* **92**, 347–356 (1989).
5. Green, D. H. & Wallace, M. E. *Nature* **336**, 459–462 (1988).
6. Yaxley, G. M., Crawford, A. J. & Green, D. H. *Earth planet. Sci. Lett.* **107**, 305–317 (1991).
7. Daturia, J. M., Dupuy, C., Takherist, D. & Dostal, J. *Contrib. Miner. Petrol.* **111**, 37–52 (1992).
8. Rudnick, R. L., McDonough, W. F. & Chappell, B. W. *Earth planet. Sci. Lett.* **114**, 463–475 (1993).
9. Wyllie, P. J. & Huang, W. L. *Contrib. Miner. Petrol.* **54**, 79–107 (1976).
10. Eggler, D. H. *Am. J. Sci.* **278**, 305–343 (1978).
11. Brey, G. et al. *Earth planet. Sci. Lett.* **62**, 63–74 (1983).
12. Brenan, J. M. & Watson, E. B. *Geochim. cosmochim. Acta* **55**, 2203–2214 (1991).
13. Green, T. H., Adam, J. & Sie, S. H. *Miner. Petrol.* **46**, 179–184 (1992).
14. Hart, S. R. & Dunn, T. *Contrib. Miner. Petrol.* **113**, 1–18 (1992).
15. Hauri, E. H., Wagner, T. P. & Grove, T. L. *Chem. Geol.* (submitted).

16. Nelson, D. R., Chivas, A. R., Chappell, B. W. & McCulloch, M. T. *Geochim. cosmochim. Acta* **52**, 1–17 (1988).
17. Palacz, Z. A. & Saunders, A. D. *Earth planet. Sci. Lett.* **79**, 270–280 (1986).
18. Wright, E. thesis, Scripps Inst. Oceanogr. (1986).
19. Chauvel, C., Hofmann, A. W. & Vidal, P. *Earth planet. Sci. Lett.* **110**, 99–119 (1992).
20. Ionov, D. A., Hofmann, A. W. & Shimizu, N. *J. Petrol.* (in the press).
21. Watson, E. B. *Contrib. Miner. Petrol.* **56**, 119–134 (1976).
22. Schiano, P., Ciocchiatti, R., Mattioli, N., Weis, D., Shimizu, N. *EOS* **74**, 320 (1993).
23. Wright, E. & White, W. M. *Earth planet. Sci. Lett.* **81**, 151–162 (1987).
24. Farley, K. A., Natland, J. & Craig, H. *Earth planet. Sci. Lett.* **111**, 183–217 (1992).
25. Zindler, A. & Hart, S. R. A. *Rev. Earth planet. Sci.* **14**, 493–571 (1986).
26. Martin, C. E. *Geochim. cosmochim. Acta* **55**, 1421–1434 (1991).
27. Luck, J.-M. & Allegre, C. J. *Earth planet. Sci. Lett.* **107**, 406–415 (1992).
28. Wallace, M. E. & Green, D. H. *Nature* **335**, 343–346 (1988).
29. Baker, M. & Wyllie, P. J. *Nature* **346**, 168–170 (1990).
30. Hauri, E. H. & Hart, S. R. *Earth planet. Sci. Lett.* **114**, 353–371 (1993).
31. Hofmann, A. W. & White, W. M. *Carnegie Inst. Wash. Yb.* **79**, 477–484 (1980).
32. Chase, C. G. *Earth planet. Sci. Lett.* **52**, 277–284 (1981).
33. Davidson, J. P. *Nature* **306**, 253–256 (1983).
34. White, W. M., Dupre, B. & Vidal, P. *Geochim. cosmochim. Acta* **49**, 1875–1886 (1985).
35. White, W. M. & Dupre, B. *J. geophys. Res.* **91B**, 5927–5941 (1986).
36. Woodhead, J. D., Greenwood, P., Harmon, R. S. & Stoffers, P. *Nature* **362**, 809–813 (1993).
37. Anders, E. & Grevesse, N. *Geochim. cosmochim. Acta* **53**, 197–214 (1989).
38. Liotard, J.-M. & Barszcz, H. G. *C.r. hebd. Séanc. Acad. Sci., Paris* **308**, 1261–1266 (1989).

ACKNOWLEDGEMENTS. We thank J. Blusztajn, P. Kelemen and H. Barszczus for discussions, and D. Green, R. Rudnick and A. Hofmann for reviews. This research was supported by the US NSF.

DNA topoisomerase I is involved in both repression and activation of transcription

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Reconstituted transcription reactions containing the seven general transcription factors, in addition to RNA polymerase II, respond poorly to transcriptional activators. Two factors, Dr₂ and ACF, necessary for high levels of transcription in response to an activator have been identified. ACF can enhance basal and activated transcription. Dr₂ represses basal transcription, but this can be overcome by transcriptional activators or TFI_{II}A. Dr₂ is human DNA topoisomerase I. The DNA relaxation activity of topoisomerase I is dispensable for transcriptional repression. The effect of Dr₂ is specific for TATA-box-containing promoters and is mediated by the TATA-binding protein.

EARLY studies established that certain proteins can activate transcription¹. Although the mechanism(s) of this activation is unclear, it has been presumed that activator proteins work by communicating with and influencing the basal transcription machinery². Recent studies have shown specific and direct interactions between regulatory factors and some of the general transcription factors (GTFs). Specifically, a direct interaction between an acidic activator and TFI_{II}B³, TFI_{II}H (D.R. and J. Greenblatt, unpublished result) and the TATA-binding protein (TBP) subunit of TFI_{II}D⁴ have been demonstrated. These interactions appear to be part of the activation process, as mutations in the acidic domain of an activator, which weaken or eliminate activation of transcription, also reduce the extent of interaction with the particular GTF^{3,4}. Moreover, mutations of specific residues in TFI_{II}B, which eliminate the interaction with an acidic activator, abolish activated transcription, but have little effect on the ability of TFI_{II}B to participate in basal transcription⁵. Despite these findings, physiological levels of activation cannot be reproduced in a reconstituted transcription system composed of an activator, TFI_{II}D, and the remaining GTFs. Thus, contact between components of the basal machinery and an activator is not sufficient to stimulate transcription; additional factors appear to be necessary.

To elucidate the mechanism(s) operating to activate transcription, we analysed the factors necessary for activation *in vitro*. We determined that the response to an acidic activator requires two protein fractions, ACF and Dr₂, in addition to TFI_{II}D, the

GTFs and RNA polymerase II. In the absence of an activator, Dr₂ represses basal transcription, but in the presence of an activator, Dr₂ stimulates this response. Dr₂ was purified to homogeneity from HeLa cells and identified as topoisomerase I.

Dr₂ and ACF necessary for optimal transcription

To analyse the factors required for activation of transcription *in vitro*, reactions were reconstituted as follows: a circular DNA template containing five GAL4-DNA-binding sites upstream of the TATA motif of the adenovirus major late promoter (Ad-MLP), was mixed with a second template lacking GAL4-DNA-binding sites. The addition of increasing amounts of a fusion protein composed of the GAL4-DNA-binding domain and an acidic domain (GAL4-AH)⁶ to a transcription assay containing both templates, TFI_{II}D, the GTFs and RNA polymerase II resulted in low, non-physiological levels of activated transcription from the template containing the GAL4-DNA-binding sites (Fig. 1a, compare lane 1 with 2–4). The template lacking GAL4 sites was unaffected. The addition of the Dr₂ protein fraction (see Fig. 2 legend) resulted in repression of basal transcription and stimulation of activated transcription (Fig. 1a, compare lane 5 with 1 and 3). The addition of the ACF protein fraction resulted in further stimulation of transcription from the activator-responsive template (lanes 6 and 7). Under these conditions Dr₂ repressed basal transcription.

The individual effects of Dr₂ and ACF in transcription, in the presence and absence of activator and with an excess amount of GTFs are shown in Fig. 1b. The addition of increasing amounts of Dr₂ to a transcription assay devoid of an activator resulted

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in repression (lanes 1–4). With activator, Dr₂ repressed only transcription from the DNA lacking the GAL4 recognition elements, whereas the template containing the GAL4 sites was stimulated (compare lane 9 with 10–12). Under these conditions the net effect of the activator was about a 20-fold stimulation of transcription (compare lane 4 with 12). The addition of ACF to reactions lacking Dr₂ and activator resulted in a modest stimulation of transcription at low ACF concentrations and a moderate inhibition from both templates at higher concentrations (Fig. 1b). The addition of both Dr₂ and ACF repressed transcription from both templates in the absence of the activator (lane 8), yet in its presence the activator-responsive template was stimulated, with the net effect of GAL4-AH being a 40-fold enhancement of transcription (compare lanes 8 and 17).

We demonstrated that the GAL4(1–94) DNA-binding domain by itself could not overcome repression of transcription (Fig. 1c, lane 4), indicating that an activating domain is required. Furthermore, the antirepression phenomenon was not specific for GAL4-AH, as GAL4-VP16 (lane 8), or glutamine-rich (lane 10) and proline-rich (lane 12) activators were capable of neutralizing the repressing effect of Dr₂.

Dr₂ contains DNA topoisomerase I activity

Dr₂ activity was purified to apparent homogeneity and found to reside in a polypeptide of apparent *M_r* 97K (Fig. 2a). Renaturation experiments with the 97K polypeptide eluted from SDS-PAGE confirmed this observation (data not shown). Highly purified Dr₂ was transcriptionally active (Fig. 2b).

Digestion of Dr₂ with trypsin yielded eight polypeptides whose amino-acid sequences are indicated in Fig. 2. Comparison of

these sequences with those in the Genebank database revealed that all the peptides are present in human topoisomerase I (hTopo I)⁷. This led us to investigate whether the purified preparation of Dr₂ contained Topo I activity. Consistently, Dr₂ could relax supercoiled DNA (Fig. 2c).

Dr₂ and topoisomerase I activities

To analyse further whether Dr₂ and Topo I activities were contained in the same polypeptide, a recombinant form of hTopo I was purified from baculovirus-infected insect cell extracts. Silver staining of a SDS-PAGE revealed the presence of 97K and 77K polypeptides in this sample. These polypeptides were absent in the extract derived from wild-type baculovirus-infected cells (Fig. 3a). The 97K rhTopo I polypeptide comigrated on SDS-PAGE with Dr₂ (Fig. 3a) and both polypeptides reacted with

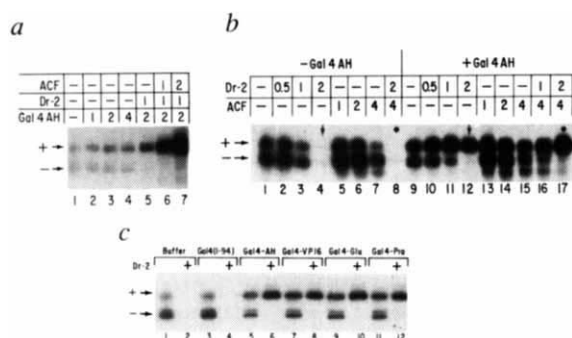


FIG. 1 ACF and Dr₂ are necessary to achieve optimal response to an activator. **a**, Transcription reactions contained a DNA template with five GAL4-binding sites upstream of the TATA motif directing transcription of a 380 nucleotides G-less cassette (+ transcripts) and a second DNA template lacking GAL4-binding sites and producing a 330-nucleotide transcript (– transcript). Reactions also contained the different GTFs and RNAPII. In addition, reactions contained GAL4-AH (50 ng), Dr₂ (200 ng), and ACF (275 ng as 1 × concentration) as indicated. **b**, The individual effect of increasing concentrations of ACF and Dr₂ was analysed in the absence and presence of the activator GAL4-AH. Reactions were as described in **a**, but the concentration of the GTFs was doubled. **c**, Different activators, as indicated, were analysed for their ability to overcome Dr₂-mediated inhibition. The amount of activator added to the reactions was standardized by measuring DNA-binding activity (data not shown).

METHODS. Transcription reactions were as previously described³⁸ and contained 200 ng of each of the DNA molecules, RNAPII and the different GTFs: IIA/IIJ, IIB, IID, rIE, IIF, and IIH, as previously described³⁸. The RNA products were analysed on a polyacrylamide-urea gel. The synthetic transcriptional activators GAL4-AH, GAL4-VP16, GAL4-Glu, GAL4-Pro and the DNA-binding domain of GAL4(1–94) were purified as described^{6,39–41}. All proteins were kept in buffer C (20 mM Tris-HCl, pH 7.9, 10% glycerol, 1 mM ethylenediamine tetra-acetic acid, 0.01% Tween20, 0.2 mM phenylmethylsulphonyl fluoride and 5 mM β-mercaptoethanol) containing 0.1 M KCl. Dr₂ and ACF were purified from the previously described phosphocellulose 1 M fraction (see Fig. 2 legend).

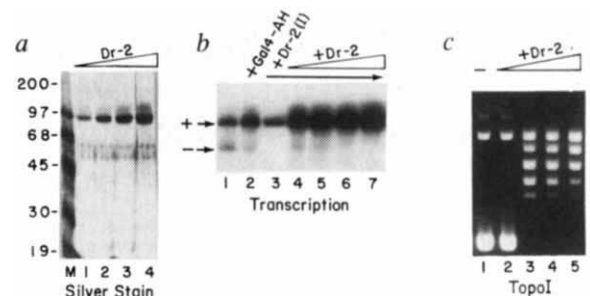


FIG. 2 Identification of Dr₂. Aliquots (75, 100, 150 and 200 ng) of the μ -Mono S Dr₂ protein pool were analysed by SDS-PAGE followed by silver staining (**a**) and transcription (**b**). Transcription reactions were as described in Fig. 1b. Lane 3 indicates a reaction containing an aliquot of the Dr₂-phenyl-Superose protein pool (400 ng). The Topo I activity of the μ -Mono S pool was examined (**c**). The dots to the right of **c** indicate DNA topoisomers. The μ -Mono S Dr₂ fraction was further purified on a μ -reverse-phase chromatography (RPC) column and a single peak was recovered. This material was used for amino-acid analysis and trypsin digestion. The peptides obtained after trypsinization were separated on an RPC column and sequenced by Edman degradation as described⁴². **METHODS.** The 1.0 M phosphocellulose fraction derived from HeLa cell nuclear extracts was fractionated on a CM-Sephacrose column as previously described¹⁷. Fractions containing TFIIID and Dr₂/ACF activities were pooled (315 mg), dialysed against buffer C (50 mM KCl) and loaded onto a 60-ml DEAE-52 column. About 80% of the Dr₂/ACF activities passed through the column. This fraction also contained ~30% of the input TFIIID activity. The DEAE-52 flow-through (163 mg) was loaded onto a MonoQ column (Pharmacia, HR10/10). The flow-through (51 mg) containing Dr₂/ACF was further purified by chromatography on a phenyl-Superose column (Pharmacia, HR10/10). The column was equilibrated with buffer C containing 1.2 M ammonium sulphate ((NH₄)₂SO₄). Proteins were eluted with a linear gradient (1.0–0 M) of (NH₄)₂SO₄ in buffer C. This resulted in the separation of Dr₂ and ACF. Dr₂ activity eluted at 0.7 M (NH₄)₂SO₄, whereas ACF activity eluted at 0.3 M (NH₄)₂SO₄. The phenyl-Superose Dr₂ fraction (810 μ g) was further purified on a μ -MonoS column (SMART-Pharmacia-LKB) equilibrated with buffer C containing 50 mM KCl. The Dr₂ activity was eluted with a linear gradient between 50–500 mM KCl. The fractions containing Dr₂ activity (250–300 mM KCl) were pooled (9 μ g) and further purified on a μ -RPC column (SMART-Pharmacia-LKB). The column was developed with a linear gradient of acetonitrile (0–100%) containing 0.1% trifluoroacetic acid; Dr₂ polypeptide elutes at 65% acetonitrile. The Dr₂ polypeptide (95 pmol) was digested with trypsin and the amino-acid sequence of eight peptides were as follows (in single-letter code): (1) AVALYFDKLALR, (2) TYNASTLQQQLK, (3) AVQRLEEQLMK, (4) NIITNLSK, (5) AVAILCNHQR, (6) IKGEKDWQKYETAR, (7) SMMNLQTK, (8) NFFK. Topoisomerase I assays were as follows: supercoiled DNA (0.3 μ g) was incubated with 1, 2, 4 and 8 μ l of Dr₂ μ -MonoS (diluted 1/3,200) protein fraction for 5 min at 30 °C. The 10- μ l reactions contained 20 mM HEPES, pH 7.9, 8 mM MgCl₂, 2.5 mM (NH₄)₂SO₄, 2% polyethylene glycol 8000 and 50 mM EDTA. These reactions were stopped with 2 μ l 1% SDS, 30% glycerol, 0.25% xylene xienol and bromophenol blue, and the products were analysed on a 1% agarose gel followed by staining with ethidium bromide.

anti-Topo I antibodies (Fig. 3b). The 77K rhTopo I polypeptide also reacted with the antibodies. A similar polypeptide has been found in human cell extracts and was shown to be a proteolysed form of hTopo I lacking N-terminal residues⁸. Fractions containing the recombinant 97K and 77K polypeptides, but not fractions derived from wild-type baculovirus-infected cells, contained Dr₂ activity. This was analysed by measuring the ability of the fraction to repress basal transcription (Fig. 3c). Both the 77K and 97K rhTopo I polypeptides contained Dr₂ activity (data not shown).

It was important to analyse whether the DNA relaxation activity of hTopo I was necessary for Dr₂ function. Tyrosine at position 723 in the active site of hTopo I is essential for DNA relaxation activity⁹. A mutant hTopo I gene was engineered with tyrosine 723 substituted by phenylalanine (Y723F). This baculovirus-expressed form of hTopo I was purified and analysed for Dr₂ activity. In agreement with previous studies⁹⁻¹¹, the mutant rhTopo I protein was inactive in DNA relaxation (data not shown), yet this protein was still capable of repressing basal transcription (Fig. 3d, lane 4) and yielding high levels of activated transcription in the presence of ACF (lane 6). ACF, at the concentration used and in the absence of Dr₂, failed to stimulate the response to the activator (lane 7).

Gene-specific repression of transcription

Having found that hTopo I is a repressor of basal transcription, we analysed the specificity of these observations. It is possible that the observed effect was a result of the DNA-binding activity associated with hTopo I¹². We thus analysed whether Dr₂ activity is present in topoisomerases derived from different sources. Vaccinia virus, *Escherichia coli*, and yeast Topo I proteins were unable to repress basal transcription (Fig. 4a), suggesting that the Dr₂ activity present in hTopo I is unlikely to result solely from DNA binding. This agrees with our analysis indicating that optimal Dr₂ activity requires about five molecules of hTopo I per molecule of DNA, a quantity insufficient to cover the DNA completely and account for total repression of basal transcription.

The specificity of Dr₂ was further scrutinized by analysing whether different class II promoters could be repressed by Dr₂. The promoters chosen were the Ad-E4 and the TATA-less β -DNA polymerase promoters. The minimal elements required for transcription from these promoters were placed downstream of five GAL4 recognition sites. Transcription used nuclear extracts supplemented with GAL4-AH and Dr₂. Basal transcription from these promoters was below detectable levels; however, the addition of GAL4-AH resulted in transcription (Fig. 4b, compare lanes 1 and 2). The addition of increasing concentrations of Dr₂ resulted in inhibition of transcription from the Ad-E4 promoter; however, transcription directed by the β -DNA polymerase promoter was unaffected (lanes 3–5). We reason that these promoters respond differently to Dr₂ because they use different pathways to promote preinitiation complex formation¹³. In the case of the Ad-E4 promoter, transcription is directed by the TATA motif. Transcription from the TATA-less β -DNA polymerase promoter is directed by the initiator (Inr), which results in assembly of a complex responsive to GAL4-AH yet unresponsive to Dr₂. To substantiate this observation further, the prototype Ad-MLP with (pG₆TI) and without (pG₆I) a TATA motif and five Sp1-binding sites was analysed. Consistently, TATA-dependent transcription, but not Inr-directed transcription, was inhibited by topoisomerase I (Fig. 4c). These results indicate that the effect of Dr₂ is not a consequence of DNA binding, and more importantly, they suggest that the inhibitory function of Dr₂ is promoter-specific.

TBP mediates Dr₂ effect on transcription

To analyse the step at which Dr₂ functions during complex formation, we attempted to isolate a preinitiation complex intermediate refractory to Dr₂ inhibition. Subsets of GTFs required

to direct formation of transcription-competent complexes (TFIID, -11A and -11B) were incubated with DNA before the addition of Dr₂, as illustrated in Fig. 5a. The incubation of TFIID, TFIIA, TFIIB and Dr₂ with DNA resulted, as expected, in inhibition of basal transcription (lane 3). However, inhibition could not be observed if Dr₂ was added after the formation of the D·A complex (lane 5). TFIIB was without effect (lane 4), and the incubation of TFIID and DNA, in the absence of TFIIA, was not sufficient to overcome Dr₂ inhibition (lane 6).

Although TFIIA is not required for basal transcription when TBP is used in a reconstituted system¹⁴, our previous observations demonstrated that TFIIA was necessary to obtain comparable levels of transcription when the TFIID complex was implemented in the assay¹⁴. This result led us to suggest that TFIIA may function to remove negative regulators present in the TFIID complex¹⁴. To test this hypothesis, we investigated whether Dr₂ is present in the TFIID complex.

TFIID was purified from HeLa cells and from a cell line containing a stably integrated epitope-tagged TBP (eTFIID)¹⁵. Both forms of TFIID were fractionated through a protein A column containing antibodies recognizing the epitope of eTFIID. Proteins were eluted from the columns with increasing salt washes. The presence of Dr₂ was determined by measuring Topo I enzymatic activity and by western blot using anti-Topo I antibodies. The presence of epitope-tagged TFIID was also investi-

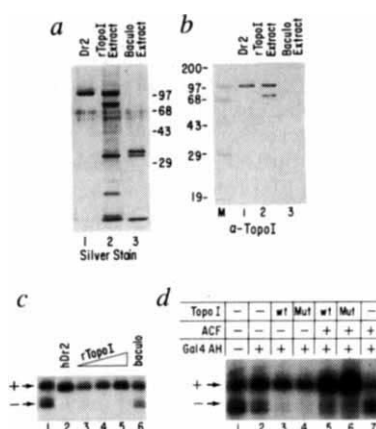
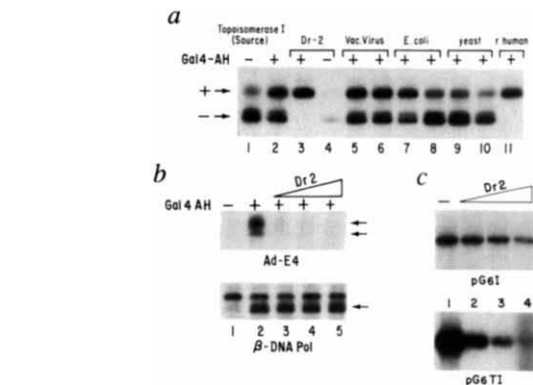


FIG. 3 Recombinant hTopo I contains Dr₂ activity. **a**, Silver staining of an SDS-polyacrylamide gel representing highly purified Dr₂ (lane 1), proteins isolated from SF9 insect cells infected with a hTopo I recombinant baculovirus (lane 2), or a wild-type baculovirus (lane 3). **b**, Western blot analysis using anti-hTopo I antibodies; lanes as in **a**, with BRL prestained M_r markers (lane M). **c**, Dr₂ (μ -Mono S protein fraction, 200 ng, lane 2), and hTopo I isolated from hTopo I-baculovirus-infected SF9 cells (200, 300 and 400 ng, lanes 3–5), and proteins (200 ng) from the wild-type (wt)-baculovirus-infected SF9 cells (lane 6), were analysed for their ability to repress basal transcription, as described in Fig. 1. **d**, Recombinant wild-type and mutant (Y723F) hTopo I proteins (400 ng each) were analysed in basal and activated transcription, in the presence of ACF (550 ng), as indicated on the top of the panel. Transcription assays are described in the legend to Fig. 1.

METHODS. Recombinant hTopo I was generated using a baculovirus expression system. Extracts were prepared from hTopo I-baculovirus- or wild-type baculovirus-infected SF9 insect cells and non-rhTopo I proteins were precipitated with polyethyleneglycol in high salt (K.R.M. et al., manuscript in preparation). The supernatant fraction was dialysed against buffer C containing 100 mM KCl, resulting in the precipitation of rhTopo I. rhTopo I was recovered by centrifugation at 10,000g for 10 min and resuspended in 6 M GuHCl. The denatured proteins were renatured by dilution to 0.1 M guanidinium-HCl with buffer C containing 100 mM KCl. A similar extract was prepared with recombinant baculovirus carrying a single substitution (Y723F) in the Topo I gene (K.R.M. et al., manuscript in preparation). Because the mutant rhTopo I is deficient in DNA relaxation⁹, silver staining of SDS-PAGE and western blot analysis⁹, were used to analyse the integrity and the concentration of the recombinant proteins (data not shown).

FIG. 4 Dr₂/hTopo I-dependent repression of transcription is species- and promoter-specific. **a**, Topoisomerases from different sources were used as source of Dr₂ in transcription. Similar (odd lanes) or twice the amounts (even lanes) of vaccinia virus⁴³, *E. coli*⁴⁴ and *S. cerevisiae*¹¹ topoisomerases, relative to the hTopo I DNA-relaxing activity in Dr₂, were tested on transcription as described for Fig. 1 (lanes 5–10). Positive controls included Dr₂ (μ -MonoS, 200 ng) (lanes 3 and 4) and recombinant hTopo I (400 ng, Lane 11). The quantification of the topoisomerase DNA-relaxing activity was done using serial dilutions of the different samples, as shown in Fig. 2c with the Dr₂ fraction. **b**, Primer extension analysis of transcription reactions directed by the Ad-E4 (pG5D38E4CAT⁴⁵; gift from M. Green) and the β -DNA polymerase (pG5 β -polCAT; L. Weis and D.R., manuscript in preparation) promoters, both containing 5 GAL4 DNA-binding sites upstream from the TATA motif. Reactions were done as previously described⁴⁶ using HeLa nuclear extracts (210 μ g) supplemented with GAL4-AH (1.8 μ g, lanes 2–5) and increasing amounts of Dr₂ (lanes 3–5, 0.6, 0.9 and 1.2 μ g, respectively). **c**, Reactions as in **b**, but containing the Ad-MLP with six Sp1-binding sites upstream of the TATA motif (pG6TI), or a similar DNA construct in which the TATA motif was mutated (pG61) and thus



transcription was directed by the initiator. These DNA templates directed transcription of a G-less cassette⁴⁷. The nuclear extract was supplemented with Dr₂ as described in **b** (lanes 2–4).

gated using anti-epitope antibodies. Topo I was detected in the 1.0 M KCl phosphocellulose TFIID and eTFIID protein fractions (Fig. 5b, c, lanes 1 and 2), as well as in the flow-through of the protein A columns (Fig. 5b, c, lanes 3 and 4). However, Topo I from the eTFIID fraction was also retained by the protein A column and was eluted with 0.2 and 0.5 M KCl washes (Fig. 5b, c, lanes 8 and 10). Topo I present in the non-epitope tagged TFIID fraction was not retained by the protein A column (Fig. 5b, c, lanes 7 and 9). These results indicate that Dr₂/hTopo I is associated with the TFIID complex. To analyse whether hTopo I directly interacts with TBP, a FAR-western analysis was done using rhTopo I. The interaction was analysed using human biotinylated TBP. These studies demonstrate that there is a direct interaction of Topo I with TBP (Fig. 5d, lane 2). The interaction is specific, as it was not evident in samples lacking

rhTopo I (lanes 3 and 4) nor in samples blotted with avidin-alkaline phosphatase without biotinylated TBP (data not shown).

The results presented above indicate that the effect of Dr₂ in transcription is mediated by an interaction with TBP. Moreover, the functional analysis (Fig. 5a) suggests that TFIIA and Dr₂ may counteract each other's function. In light of these results and our previous analysis of the role of TFIIA in transcription¹⁴, we analysed the effect of Dr₂ and TFIIA in reactions reconstituted with TBP. Transcription activity could be demonstrated in the absence of TFIIA, provided that TBP was used instead of TFIID (Fig. 6a, lane 1). The addition of Dr₂ resulted in an inhibition that could be overcome by TFIIA (Fig. 6a). Moreover, the preincubation of TBP and TFIIA, before addition of Dr₂, resulted in a reaction resistant to inhibition (Fig. 6b). Thus,

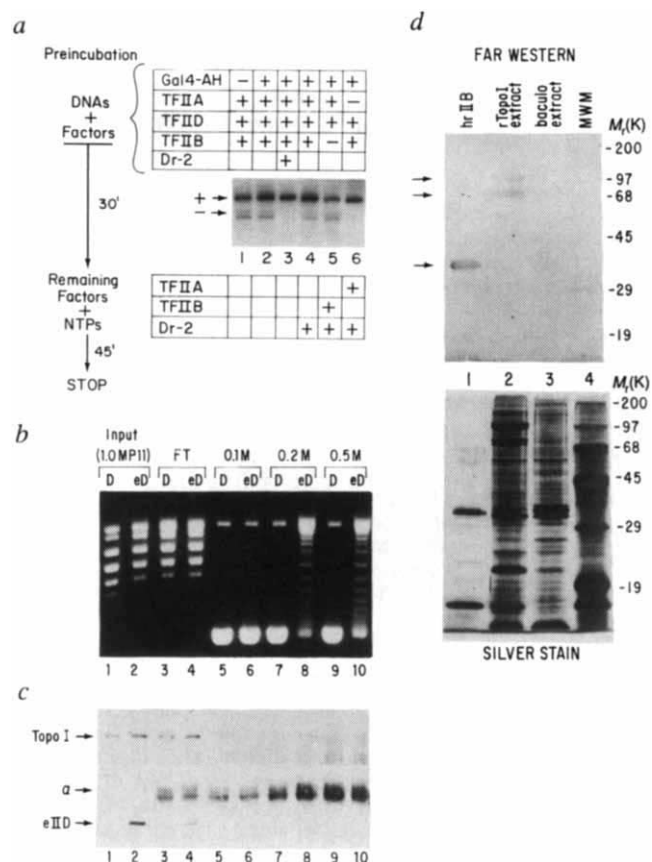
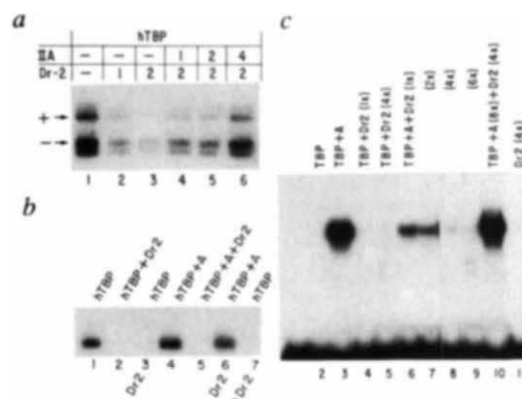


FIG. 5 Dr₂/hTopo I interacts with TBP preventing the formation of a transcription-competent complex. **a**, The DA complex is refractory to Dr₂ inhibition. Transcription reactions were done as described for Fig. 1, but modified as shown on the left-hand side of the panel. A combination of GTFs and GAL4-AH (as shown on the upper part of the panel) were preincubated with DNA for 30 min at 30 °C before addition of NTPs and the rest of the GTFs (as described in the lower part of the panel). hTopo I was added to the transcription reactions during (lane 3) or after preincubation (lanes 4–6). **b**, **c**, Dr₂/hTopo I interaction with TFIID in solution. Nuclear extracts from normal and stably transformed HeLa cells containing the epitope-tagged TFIID¹⁵, were purified on a phosphocellulose column. The 1.0 M phosphocellulose fractions were then loaded onto independent columns containing a monoclonal antibody directed against the epitope tag of TBP (YPYDVPDYA). The columns were washed extensively with buffer C (100 mM KCl) and eluted by increasing the concentration of KCl to 200 and 500 mM. The fractions obtained were assayed for DNA relaxation activity as in Fig. 2 (b), and by western blot analysis using antibodies against the epitope tag of eTFIID and hTopo I⁹ (c); **a** represents the anti-mouse IgG reacting material leaking from the immunoaffinity column. **d**, FAR-western analysis of rhTopo I using biotinylated human TBP. Proteins present in recombinant TFIIB⁴² (lane 1), extracts derived from hTopo I-baculovirus (lane 2), wt-baculovirus-infected cells (lane 3), and markers (lane 4) were separated by SDS-PAGE. One gel was silver-stained (lower part of the panel) and a duplicate was transferred to nitrocellulose. **METHODS.** Anti-epitope tag IgG monoclonal antibody, mAb12CA5 (50 μ g; Berkeley Antibody Co., Inc.), were immobilized on 50 μ l of protein A-Sepharose (Pharmacia). The FAR-western analysis was done as described³¹; however, the blocking agent was changed from 5% milk to 3% gelatin. TFIIB and Topo I (~1 μ g each) were blotted with 2 μ g ml⁻¹ biotinylated hTBP overnight at room temperature.

FIG. 6 TFIIA overcomes Dr₂/hTopo I-mediated repression. **a**, Transcription reactions were done as described for Fig. 1 with the following changes. hTBP (40 ng) was used instead of TFIID. Increasing amounts of a hydroxylapatite TFIIA fraction¹⁴ (50, 125 and 200 ng) were added to the reactions containing the highest amount of Dr₂ (150 ng, μ -MonoS). **b**, The TBP-A complex is refractory to Dr₂ inhibition. Transcription reactions were done as described for Fig. 1a, but modified as shown on the left-hand side of Fig. 5a. A combination of TBP, TFIIA and Dr₂ (as shown on the upper part of the panel) was preincubated with DNA for 30 min at 30 °C before addition of NTPs and the rest of the GTFs. Dr₂ was added to the transcription reactions during (lanes 2 and 5) or after preincubation (lanes 3, 6 and 7). **c**, Mobility shift assay measuring the effect of Dr₂ on the DA complex. DNA-binding assays were done using a ³²P-labelled Ad-MLP DNA fragment (-55 to +40) and contained hTBP (20 ng), TFIIA (25 ng, hydroxylapatite fraction) and Dr₂ (50 ng), all as 1 $\times$ concentrations, or as indicated at the top of the panel. **METHODS.** Binding assays were done in 10 μ l containing 20 mM HEPES, pH 7.9, 8 mM MgCl₂, 2.5 mM (NH₄)₂SO₄, 2% polyethylene glycol 8000, 1 μ g poly-dG/poly-dC (Pharmacia). The DNA-protein complexes were separated on a non-denaturing polyacrylamide gel as previously described^{16,17}.



TFIIA neutralizes the effect of Dr₂. This was further analysed using the gel-mobility shift assay. In agreement with previous observations^{16,17}, the addition of TBP and TFIIA to DNA-binding assays using a TATA-containing fragment resulted in the formation of the D·A complex (Fig. 6c, lane 3). In the absence of TFIIA, TBP could not produce a stable complex with the TATA motif (lane 2). Although Dr₂ interacts directly with TBP (Fig. 5d), a Dr₂-TBP-DNA complex could not be demonstrated under these conditions (Fig. 6c, lanes 4, 5); nor could Dr₂ bind stably to DNA (lane 11). Nonetheless, the simultaneous addition of Dr₂ and TFIIA to TBP-containing reactions resulted in inhibition of D·A complex formation; the extent of inhibition was proportional to the amount of Dr₂ added (lanes 6–9). This inhibition was specific, as excess TFIIA could restore the D·A complex (lane 10). These results not only present a possible mechanism for Dr₂/topo I-mediated repression of transcription, but also argue against a generic inhibition due to the nonspecific DNA-binding activity associated with Topo I¹².

Discussion

We have analysed the factors necessary to mediate activation of *in vitro* transcription mediated by RNA polymerase II. We find that in addition to the activator, TFIID, and the remaining GTFs, two other components, ACF and Dr₂, are necessary to achieve levels of activation approaching those observed *in vivo*. ACF is poorly defined and probably includes factors previously described as mediators or adaptors^{18–21}. Dr₂ was identified as hTopo I. This factor not only augmented the response to the activator, but in the absence of the activator, repressed basal transcription. The repressing activity associated with Dr₂ cannot be explained by the nonspecific DNA-binding activity associated with hTopo I¹³, as our experiments demonstrate that: (1) topoisomerases isolated from sources other than human are unable to repress the human transcription system; (2) Dr₂/hTopo I is part of the TFIID complex and specifically interacts with TBP; and (3) Dr₂ activity is specific for those promoters that contain a TATA motif. A promoter lacking this motif, such as the β -DNA polymerase promoter, or the TdT-Inr-mediated transcription, is resistant to Dr₂-mediated repression. Our observations agree with recent studies demonstrating that the tumour-suppressor p53 protein interacts with TBP to repress transcription²². Moreover, the studies of Mack and co-workers found that p53-mediated repression requires a TATA motif and that Inr-directed transcription is resistant to inhibition²³. Our studies also agree with the *in vivo* studies of Choder²⁴, who observed that when yeast cells reached stationary phase, topoisomerase I (yTopo I) can repress transcription of most, but not all, genes. We found that the DNA-relaxation activity associated with hTopo I was not required for repression of transcription, because

hTopo I(Y723F), a mutant lacking DNA relaxation activity⁹, was active in repression, as well as in enhancing the response to the activator.

Despite the observations that the human polypeptide and yeast Topo I gene can repress transcription, we found that yeast Topo I was not able to affect transcription in the human system. This is surprising, because hTopo I could interact with yeast TBP (data not shown). The molecular basis for the functional differences remains to be determined; however, precedent for this kind of observation does exist. For example, human TFIIB can interact with yeast TBP (and vice versa), but the factors (human and yeast TFIIB) are not functionally interchangeable². These observations suggest that there may be other steps during formation of a transcription-competent complex where hTopo I may play a role. This step appears to be species-specific. Indeed, our preliminary studies indicate that hTopo I can interact with RNAPII in solution and, moreover, it appears that hTopo I travels with RNAPII during elongation, as we have been able to detect topoisomerase I in ternary complexes (unpublished results).

Previous studies have demonstrated that topoisomerase I is associated with transcriptionally active class II genes²⁵. Moreover, the studies of Stewart *et al.*, suggested a functional interaction between Topo I and RNAPII during transcription elongation of the *fos* gene²⁶. It is tempting to postulate that hTopo I is loaded onto the transcription complex by interacting with the TFIID complex. In the absence of the activator, this interaction results in repression of transcription. But, in the presence of the activator, hTopo I is translocated from the TFIID complex to the elongating complex. This might permit effective elongation by removing the superhelical tension induced by the elongation process²⁷. Although this model is attractive, it is not supported by studies in yeast, as the deletion of the Topo I gene had no effect on cell viability^{28,29}. Studies in higher eukaryotes, however, have found that the Topo I gene is essential for development in *Drosophila*³⁰.

We have demonstrated that Dr₂ represses transcription *in vitro*, and that in the absence of an activator, TFIIA can overcome this repression. Moreover, we found that the requirement for TFIIA is dictated by the presence of Dr₂/hTopo I. These observations agree with studies by Cortes *et al.*, who postulated that a function of TFIIA in transcription is to remove repressors present in the TFIID complex¹⁴. Our observations also agree with the demonstration that TFIIA can overcome the negative effect on transcription of Dr1³¹, NC1, NC2 and DBF4^{32,33}. But Dr₂ differs from the above repressors in its failure to form a complex with TBP and DNA on mobility shift assays. The functional relationship between these repressors of RNAPII transcription remains to be elucidated. It is clear, however, from

this and other studies^{34–37} that the process of transcription activation involves at least two independent, but interrelated steps. The initial step involves the removal of molecules that maintain genes in a silent state, a process known as antirepression³⁶. The second step represents true activation, in which the levels of

expression of particular genes are increased well above basal levels. It is likely that these distinct events are coupled and that a family of ubiquitous activators operates to remove repressors, thereby setting specific genes into a state responsive to gene-specific activators. □

Received 24 March; accepted 28 July 1993.

1. Ptashne, M. & Gann, A. F. *Nature* **346**, 329–331 (1990).
2. Zavel, L. & Reinberg, D. *Progress in Nucleic Acid Research and Molecular Biology* Vol. 44 (eds Cohn, W. E. & Moldave, K.) 67–108 (Academic, San Diego, 1993).
3. Lin, Y.-S., Ha, I., Maldonado, E., Reinberg, D. & Green, M. R. *Nature* **353**, 569–571 (1991).
4. Ingles, C. J., Shales, M., Cress, W. D., Triezenberg, S. J. & Greenblatt, J. *Nature* **351**, 588–590 (1991).
5. Roberts, S. G. E., Ha, I., Maldonado, E., Reinberg, D. & Green, M. R. *Nature* **363**, 741–744 (1993).
6. Lin, Y.-S., Carey, M. F., Ptashne, M. & Green, M. R. *Cell* **54**, 659–664 (1988).
7. D'Arpa, P. et al. *Proc. natn. Acad. Sci. U.S.A.* **85**, 2543–2547 (1988).
8. Liu, L. F. & Miller, K. G. *Proc. natn. Acad. Sci. U.S.A.* **78**, 3487–3491 (1981).
9. Madden, K. R. & Champoux, J. J. *Cancer Res.* **52**, 525–532 (1992).
10. Eng, W., Pandit, S. D. & Sternglanz, R. *J. biol. Chem.* **164**, 13373–13376 (1989).
11. Lynn, R. M., Bjornsti, M., Caron, P. R. & Wang, J. C. *Proc. natn. Acad. Sci. U.S.A.* **86**, 3559–3563 (1989).
12. Wang, J. A. *Rev. Biochem.* **54**, 665–697 (1985).
13. Weis, L. & Reinberg, D. *FASEB J.* **6**, 3300–3309 (1992).
14. Cortes, P., Flores, O. & Reinberg, D. *Molec. cell. Biol.* **12**, 413–421 (1992).
15. Zhou, Q., Lieberman, P. M., Boeyer, T. G. & Berk, A. J. *Genes Dev.* **6**, 1964–1974 (1992).
16. Buratowski, S., Hahn, S., Guarente, L. & Sharp, P. A. *Cell* **56**, 549–561 (1989).
17. Maldonado, E., Ha, I., Cortes, P., Weis, L. & Reinberg, D. *Molec. cell. Biol.* **10**, 6335–6347 (1990).
18. Berger, S. L. et al. *Cell* **70**, 251–265 (1992).
19. Kelleher, R. J. I., Flanagan, P. M. & Kornberg, R. D. *Cell* **61**, 1209–1215 (1990).
20. Lewin, B. *Cell* **61**, 1161–1164 (1990).
21. Gill, G. & Tjian, R. *Curr. Opin. Genet. Dev.* **2**, 236–242 (1992).
22. Seto, E. et al. *Proc. natn. Acad. Sci. U.S.A.* **89**, 12028–12032 (1992).
23. Mack, D. H., Vartikar, J., Pipas, J. M. & Laimins, L. A. *Nature* **363**, 281–283 (1993).
24. Choder, M. *Genes Dev.* **5**, 2315–2326 (1991).
25. Fleischmann, G. et al. *Proc. natn. Acad. Sci. U.S.A.* **81**, 6958–6962 (1984).
26. Stewart, A. F., Herrera, R. E. & Nordheim, A. *Cell* **60**, 141–149 (1990).
27. Liu, L. F. & Wang, J. C. *Proc. natn. Acad. Sci. U.S.A.* **84**, 7024–7027 (1987).
28. Thrash, C., Bankier, A. C., Barrel, B. G. & Sternglanz, R. *Proc. natn. Acad. Sci. U.S.A.* **82**, 4374–4378 (1985).
29. Goto, T. & Wang, J. C. *Proc. natn. Acad. Sci. U.S.A.* **82**, 7178–7182 (1985).
30. Lee, P. L., Brown, S. D., Chen, A. & Hsieh, T.-H. *Proc. natn. Acad. Sci. U.S.A.* **90**, 6656–6660 (1993).
31. Inostroza, J. A., Mermelstein, F. H., Ha, I., Lane, W. S. & Reinberg, D. *Cell* **70**, 477–489 (1992).
32. Meisterernst, M. & Roeder, R. G. *Cell* **67**, 557–567 (1991).
33. Meisterernst, M., Roy, A. L., Lieu, H. M. & Roeder, R. G. *Cell* **66**, 981–993 (1991).
34. Laybourn, P. J. & Kadonaga, J. K. *Science* **254**, 238–245 (1991).
35. Workman, J. L., Taylor, I. C. A. & Kingston, R. E. *Cell* **64**, 533–544 (1991).
36. Croston, G. E., Kerrigan, L. A., Lira, L. M., Marshak, D. R. & Kadonaga, J. T. *Science* **151**, 643–649 (1991).
37. Croston, G. E., Laybourn, P. J., Paranjape, S. M. & Kadonaga, J. T. *Genes Dev.* **6**, 2270–2281 (1992).
38. Lu, H., Flores, O., Weinmann, R. & Reinberg, D. *Proc. natn. Acad. Sci. U.S.A.* **88**, 10004–10008 (1991).
39. Chasman, D. I., Leatherwood, J., Carey, M., Ptashne, M. & Kornberg, R. D. *Molec. cell. Biol.* **9**, 4746–4749 (1989).
40. Lillie, J. W. & Green, M. R. *Nature* **338**, 39–44 (1989).
41. Tanese, N., Pugh, B. F. & Tjian, R. *Genes Dev.* **5**, 2212–2224 (1991).
42. Ha, I., Lane, W. S. & Reinberg, D. *Nature* **352**, 689–695 (1991).
43. Shuman, S. J. *biol. Chem.* **266**, 1796–1803 (1991).
44. Srivenugopal, K. S., Lockshon, D. & Morris, D. R. *Biochemistry* **23**, 1899–1906 (1984).
45. Liu, F. & Green, M. R. *Cell* **61**, 1217–1224 (1990).
46. Merino, A., Buckbinder, L., Mermelstein, F. H. & Reinberg, D. *J. biol. Chem.* **264**, 21266–21276 (1989).
47. Pugh, B. F. & Tjian, R. *Cell* **61**, 1187–1197 (1990).

ACKNOWLEDGEMENTS. We thank R. Drapkin, K. J. Mariani, L. Liu, M. Green, R. Sternglanz and L. Zavel for discussion and comments on the manuscript, S. Shuman, K. J. Mariani and J. Hurwitz for the gifts of vaccinia virus, *E. coli* and human DNA topoisomerases I, respectively, M. Bjornsti and J. Wang for the yeast topoisomerase I, A. Berk for sharing before publication the cell line expressing eTFIID, R. Tjian and M. Green for the GAL4 derivative proteins and their expression vectors, M. Carey for the plasmid pG5MLT, the members of the laboratory for discussions and ideas while the work was in progress, and R. Robinson for technical assistance. This work was supported by grants from the NIH to D.R.; A.M. is the recipient of the Kirin Brewery Fellowship and D.R. is a recipient of an American Cancer Society Faculty Research Award. This article is dedicated to the memory of Dr Josef Aloni.

LETTERS TO NATURE

Supernova 1993J as a spectroscopic link between type II and type Ib supernovae

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SUPERNOVA 1993J in the nearby galaxy M81 is one of the closest—and hence brightest—supernovae to be witnessed this century. The early spectrum of SN1993J showed^{1–3} the characteristic hydrogen signature of type II supernovae, but its subsequent evolution is atypical for this class of supernova. Here we present optical and infrared spectra of SN1993J up to 43 days after outburst, which reveal the onset of the helium absorption and emission features more commonly associated with hydrogen-free type Ib supernovae. Corresponding model spectra show that the progenitor star must have possessed an unusually thin (for type II supernovae) hydrogen-rich envelope overlying a helium-rich mantle. Moreover, the supernova ejecta must have remained compositionally stratified, with little transport of the hydrogen-rich material down into the underlying helium layer, or mixing of heavier elements (such as radioactive ⁵⁶Ni) outwards. SN1993J therefore represents a transition object between hydrogen-dominated type II supernovae, and hydrogen-free, helium-dominated type Ib supernovae.

Optical spectra of SN1993J taken at 5:30 UT on 29 April and 4:05 on 9 May 1993 are shown in Fig. 1 together with a spectrum

taken 20 April 1993 (ref. 1). These correspond to 32, 43 and 23 days, respectively, after the explosion (assuming that shock break-out occurred 28 March²). All spectra show the hydrogen Balmer series in absorption. The spectrum of 20 April, in which the helium features are weak or absent, shows an apparent flat-topped H α profile in both absorption and emission. Between 20 and 29 April He I lines appeared as reported earlier³. The time for this event is probably constrained more closely by the changes in the shape of the Na I 5893-Å line observed between 22 and 26 April⁴ as the He I 5876 absorption became stronger. After the helium lines become prominent, the He I 6678-Å line appears on the flat-topped H α emission feature. The strength of the helium absorption lines increased in comparison to the strength of the hydrogen features in the ~10 days elapsed between 29 April and 9 May. Further details of the observed spectra are presented in Table 1.

Infrared spectra shown in Fig. 2 were taken at 3:50 on 3 May 1993 (37 days after shock break-out). The spectrum shows He I 1.083- μ m emission with a normal P-Cygni profile, and weak He I 2.058- μ m emission with an asymmetric absorption trough. Hydrogen (Paschen) Pa- β 1.282- μ m emission is observed with a flat-topped P-Cygni profile.

Figures 1 and 2 also present the results of a model atmosphere of the supernova at 40 days after explosion. The model is derived from the unmixed 4.0- $M_{\odot}$ (solar masses) hydrodynamic model of Shigeyama et al.⁵. In this model, the supernova is originally compact, lacking an extended hydrogen-rich envelope, and hence the model will not reproduce the observed initial peak of the SN1993J light curve²; but comparison with dynamical models with low-mass extended envelopes show that it is a reasonable representation of the density structure at later times.

The parameters that we varied to obtain the best agreement with the observations are: the mass assigned to the hydrogen-

TABLE 1 Velocities at absorption minima (km s⁻¹)

Transition	Velocity on date	
	29 April	9 May
H α	-10,362	-9,790
H β	-8,375	-8,714
H γ	-8,372	-8,704
H δ *	-8,619	-9,430
He I 4,471 Å	?	-5,552
He I 4,921 Å	-5,577	-5,531
He I 5,015 Å	-5,434	-5,434
He I 5,876 Å	-7,316	-7,224
He I 6,678 Å†	-6,940	-6,496
He I 7,065 Å‡	?	-7,010
He I 7,281 Å§	?	-5,217
Ca II 8,438, 8,542, 8,662 Å	-6,197	-5,544
Fe II 4,925 Å¶		-7,785
Fe II 5,020 Å¶		-7,817

* Blended with Ca H & K, and possibly with He I 4,026 Å.

† Blended with Na I 5,893 Å.

‡ Blended with the telluric oxygen band at 6,870 Å.

§ Blended with the telluric H₂O band at 7,280 Å.

|| Mean value of 8,438-Å and 8,542-Å absorption.

¶ Measured on spectrum of 20 April, before the onset of He I 4,921 Å, and He I 5,015 Å.

TABLE 2 Theoretical model parameters

Mass at core collapse	4.00 $M_{\odot}$		
Total ejecta mass	2.45 $M_{\odot}$		
Total ^{56}Ni mass	0.08 $M_{\odot}$ ($v < 2,700$ km s ⁻¹)		
Total luminosity	7.8×10^{41} ergs s ⁻¹ (at 40 days)		
Photosphere position*	9.2×10^{14} cm ($v = 2,650$ km s ⁻¹)		
Layer	Mass ($M_{\odot}$)	v_{max} (km s ⁻¹)	$X_e^{\dagger}$
Heavy metal	0.30	2,600	0.42
Oxygen	0.47	3,200	0.20
Helium	1.40	7,500	0.04
Hydrogen/helium	0.40	11,600	0.14
Abundances (in $M_{\odot}$)			
H 0.04	C 0.07	Mg 0.05	Ca 0.01
He 1.66	O 0.47	Si 0.09	Fe 0.06
	Na 0.00	S 0.05	

* Corresponding to electron scattering of optical depth unity.

† Average ionization fraction.

rich outer layer; the H/He ratio, X , in this layer (plus solar metals); the mass and distribution of radioactive ^{56}Ni produced in the explosion; the mixing of the ejecta (independent of the ^{56}Ni distribution); the total ejecta mass; and the maximum ejecta velocity. Model details are given in Table 2 and a discussion of the general supernova atmosphere calculations are presented elsewhere⁶.

Two conditions are necessary for emission lines to form: the electron density must be low⁷ to avoid electron quenching and substantial γ -ray heating of the regions containing helium must occur⁶. The helium lines become visible after (1) the ionized outer H/He layer recombines, allowing exposure of the underlying helium mantle; (2) increasing γ -ray heating of the helium layer; and (3) the free-electron density in the mantle has declined sufficiently because of expansion and cooling. The mass adopted here for the H/He envelope, 0.4 $M_{\odot}$, with $X=0.1$, seems to produce the correct chronology and to fit the spectrum. Larger envelope masses have not been ruled out at this stage, but a smaller envelope mass would lead to premature helium line emission. The spectrum is not very sensitive to the H/He ratio in the outermost layer at 40 days; only subtle changes in the line strengths were encountered on varying the H/He mass ratio in this layer from 1/9 to 9/1.

The radioactive material cannot be accelerated to large velocities without generating excessively strong hydrogen and helium emission lines from the outermost layers. Moreover, the initial ^{56}Ni mass cannot be large, independent of distribution; models with $M_{\text{Ni}}=0.15 M_{\odot}$ resulted in nearly fully ionized hydrogen and a continuum optical depth of unity at the base of the H/He layer at 40 days, inconsistent with the spectrum. The initial mass of ^{56}Ni was therefore more likely to be $\leq 0.1 M_{\odot}$. The remaining elements cannot be mixed without contaminating the spectrum with strong 'metal' lines, particularly [Ca II] and [O I], as can be seen by comparing the mixed and unmixed model spectra of Swartz *et al.*⁶. The 6.0- $M_{\odot}$ and 3.0- $M_{\odot}$ models of Shigeyama *et al.*⁵ have expansion velocities that are slightly too low and too high, respectively. The original 4.0- $M_{\odot}$ model⁵ contains about 0.04 $M_{\odot}$ of ejecta expanding with $v > 12,000$ km s⁻¹. This is still faster than the velocities inferred from the hydrogen- and helium-line absorption minima, but if this layer is omitted the broad red wings of the helium emission lines are not reproduced. Moreover, O I 7,774-Å [O I] 6,300-Å, and [O I] 5,577-Å emission lines are formed in the relatively hot, low-density helium and H/He layers at 40 days, and a better fit to the O I 7,774-Å feature is possible with this outermost, low-density material included. The interaction of the supernova

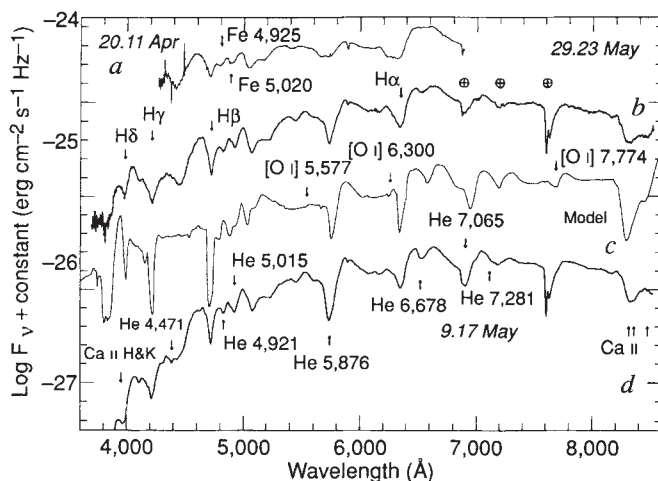


FIG. 1 Optical spectra of SN1993J. From top to bottom, the different curves are (a) The observed spectrum of SN1993J on 20 April¹ (thin line), (b) the spectrum observed at 5:30 UT on 29 April (thick line), (c) the synthetic model spectrum described in the text (thin line), (d) the spectrum observed at 4:05 UT on 9 May (thick line). The absolute fluxes (F) of the model were reddened using a mean absorption law, scaled to absorption in the visible ($A_V=0.45$). The flux scale of all spectra was arbitrarily shifted for clarity. Observations were made using the Image Grism Instrument (IGI) in spectroscopic configuration on the McDonald Observatory 2.1-m (29 April) and 0.76-m (9 May) telescopes. Feige 34 (ref. 20) HD84937, and BD+262606 (ref. 21) were used for flux calibration. The overall slopes of both spectra are systematically distorted by atmospheric differential refraction losses²² with greater loss of light in the blue portion of the observed spectra. The wavelength scale of the observed spectra has not been corrected for the motion of NGC 3031 (-49 ± 10 km s⁻¹, ref. 23). The arrows and labels close to them mark the position of blueshifted absorptions of selected lines. The Earth symbols mark the position of three strong telluric absorption features due to oxygen and water molecules.

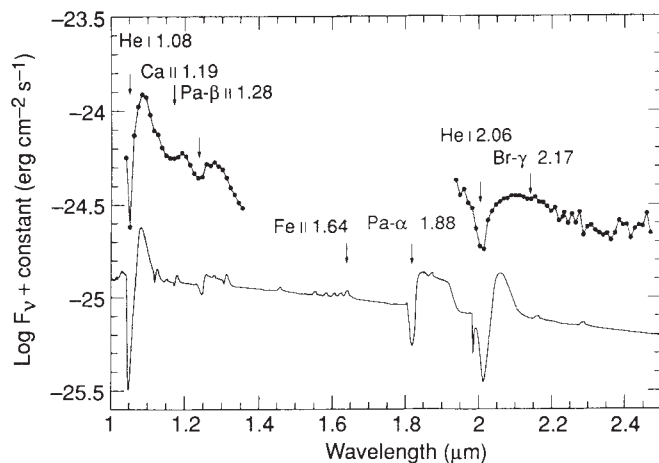


FIG. 2 Comparison of the 3 May infrared spectrum of SN1993J with the model from Fig. 1. The upper curves are the observed spectra; the solid line is the theoretical spectrum described in the text. The vertical scale indicates the flux level of the observed data, with the theoretical spectrum offset by a constant for clarity. The observations were made with the McDonald Observatory Infrared Grating Spectrometer on the 2.7-m telescope. This spectrometer is described further by Lester *et al.* (ref. 24). Spectra were taken with a low resolution grating covering the J-band (resolution ≈ 100) and the K-band (resolution ≈ 200). BS3888 (spectral type F2IV) was used for flux calibration and flat fielding assuming $J=3.24$, $K=2.99$, and a colour temperature of 6,700 K for the standard. The seeing and transparency were good but the small beam-size, combined with guiding errors, limit the absolute flux calibration to about 15%. The signal-to-noise ratio (S/N) is estimated to be ≈ 30 except towards the ends of the atmospheric windows. The region $\lambda > 2.2 \mu\text{m}$ has lower S/N (≈ 15). The blueshift from He I 1.083 μm is uncertain because the location of the minimum is sensitive to corrections for atmospheric absorption. The feature at $\lambda \sim 1.19 \mu\text{m}$ could be Ca II lines at 1.1836 and 1.1947 μm . The model gives approximately the correct flat-top profile for Pa- β while Bracket- γ is negligible. The theoretical model predicts that the He I 2.058- μm line should show a standard P-Cygni line profile, as do both the observations and model for He I 1.083 μm . The lack of distinct emission in the He I 2.058- μm observation may be due to blending with other lines not included in the model spectrum, but we note that the emission at 2.058 μm was also relatively small in SN1987A. (The notch at 1.98 μm arises from He I in the outermost zone of the model.) Arrows and symbols have the same meaning as in Fig. 1.

ejecta with a pre-existing stellar wind from the progenitor star has not been considered here, but the fact that the models favour a relatively low terminal velocity and can still tolerate a hot outermost layer, is consistent with wind-interaction models^{8,9}.

The subsequent spectral evolution of SN1993J can be estimated from these models. Over the first 100 to 150 days, Ca II and O I lines will be relatively weak and narrow relative to typical SN Ic's such as SN1987M (ref. 10) in the absence of mixing⁶. The optical helium lines will remain until ~ 100 days after explosion, but fade by about 200 days. H α and H β should be seen in absorption with weak H α emission for the first 100 days and should then also fade in the next 100 days.

The basic parameters of our model, deduced from the emergence of the helium in the spectrum—an ejecta mass, of $\sim 2 M_{\odot}$ and an outer layer of $\sim 0.4 M_{\odot}$ with a hydrogen mass fraction of $X \approx 0.1$ and a ^{56}Ni mass of $\leq 0.1 M_{\odot}$ —are similar to, but independent of, constraints from the light curve^{2,11–13} (but see ref. 14) and independent of the distance to the supernova and reddening because our results are based on modelling the spectrum and not the light curve. The ^{56}Ni mass is constrained in our model by the degree of ionization independent of the distance to SN1993J.

The helium lines in SN1993J are similar to those observed in the type Ib supernova 1984L (ref. 7) but the latter never devel-

oped hydrogen lines. This strongly suggests that SN1993J is a transition object between ordinary (plateau) type II supernovae, which retain a massive hydrogen envelope with an approximately solar abundance of helium (although the helium does not show up distinctly in the optical spectrum), and type Ib supernova, which are helium-rich^{6,7,15} but show no hydrogen at any stage of their evolution. The obvious interpretation is that mass loss of the hydrogen envelope went to completion in SN1984L and nearly to completion in SN1993J.

The spectral models predict that hydrogen and helium will no longer be observable after about 200 days. It is worth noting that the peculiar type II supernova SN1987K also showed evidence for hydrogen near maximum light but none at later times¹⁶. Moreover, closer inspection of the spectra shows that SN1987K may have begun to show lines of He I after its brightness maximum¹⁷. SN1987K may thus have been the first observed example of the class of supernovae represented by SN1993J although there are quantitative differences in the spectral developments of these two objects.

The spectral models of SN1993J also illustrate that even a small amount of hydrogen in the envelope will appear in the spectrum. This serves to emphasize that Type Ic events such as SN1987M were even more deficient in hydrogen than is SN1993J⁶, although they may contain a trace of hydrogen^{18,19}. □

Received 20 May; accepted 9 August 1993.

- Trammell, S. R., Hines, D. C. & Wheeler, J. C. *Astrophys. J.* (in the press).
- Wheeler, J. C. *et al. Astrophys. J.* (in the press).
- Filippenko, A. V. & Matheson, T. *IAU Cir. No.* 5787 (1993).
- Hu, J. Y., Li, Z. W., Jiang, X. J. & Wang, L. F. *IAU Circ. No.* 5777 (1993).
- Shigeyama, T., Nomoto, K., Tsujimoto, T. & Hashimoto, M.-A. *Astrophys. J.* **361**, L23–L27 (1990).
- Swartz, D. A., Filippenko, A. V., Nomoto, K. & Wheeler, J. C. *Astrophys. J.* **411**, 313–322 (1993).
- Harkness, R. P. *et al. Astrophys. J.* **317**, 355–367 (1987).
- Fransson, C. *Astr. Astrophys.* **133**, 264–284 (1984).
- Harkness, R. P. & Wheeler, J. C. *Proc. astr. Soc. Aust.* **7**, 431–433 (1989).
- Filippenko, A. V., Porter, A. C. & Sargent, W. L. *Astr. J.* **100**, 1575–1587 (1990).
- Nomoto, K. *et al. Nature* (in the press).
- Woosley, S. E., Eastman, R. G., Weaver, T. A. & Pinto, P. A. *Astrophys. J.* (in the press).
- Podsiadlowski, Ph., Hsu, J. J. L., Joss, P. C. & Ross, R. R. *Nature* **364**, 509–511.
- Hoflich, P., Langer, N. & Duschinger, M. *Astr. Astrophys.* (in the press).

- Lucy, I. B. *Astrophys. J.* **363**, 308–313 (1991).
- Filippenko, A. V. *Astr. J.* **96**, 1941–1948 (1988).
- Filippenko, A. V., Matheson, T. & Ho, L. C. *Astrophys. J.* (in the press).
- Jeffery, D. J., Branch, D., Filippenko, A. V. & Nomoto, K. *Astrophys. J.* **377**, L89–L92 (1991).
- Filippenko, A. V. *Astrophys. J.* **384**, L37–L40 (1992).
- Stone, R. P. S. *Astrophys. J.* **218**, 767–769 (1977).
- Oke, J. B. & Gunn, J. E. *Astrophys. J.* **266**, 713–717 (1983).
- Filippenko, A. V. *Publ. astr. Soc. Pacif.* **94**, 715–721 (1982).
- de Vaucouleurs, G. *et al. Third Reference Catalogue of Galaxies* (Springer, New York, 1991).
- Lester, D. F., Carr, J., Joy, M. & Gaffney, N. *Astrophys. J.* **352**, 544–560 (1990).

ACKNOWLEDGEMENTS. We appreciate the donation of telescope time by D. Lambert, and efforts by the staff of the McDonald Observatory to incorporate SN1993J into the schedule. This research was supported in part by grants from the NSF, NASA and the R. A. Welch Foundation. D.A.S. is supported by a NAS/NRC Resident Research Associateship.

Direct observation of reptation at polymer interfaces

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ALTHOUGH the diffusion of polymer chains in the liquid state can be described over long distances by classical diffusion laws, chain entanglements make a description of the short-range behaviour more complex. In the standard 'reptation' model^{1,2} the polymer chains are considered to move in a curvilinear manner along their own contours, exhibiting snake-like motion through the entangled sea of surrounding molecules. This model successfully explains many observations³⁻¹³, yet no direct evidence for reptative motion has been reported. Here we describe the observation of reptation of molecules across the interface between two types of partially deuterated polystyrene polymers. The time evolution of the hydrogen and deuterium profiles, determined by dynamic secondary-ion mass spectrometry, can be explained only on the basis of a reptation model. Our results demonstrate that, despite being inevitably simplified, the description of polymer diffusion in terms of reptation is essentially correct.

The reptation model predicts unique interdiffusion profiles at short times¹⁴⁻¹⁶. Measurements of these profiles in a model-independent manner has, however, not been possible^{10,17}. Probably the strongest support for reptation comes from computer simulations¹⁸⁻²¹; but only a few simulations are available on chains of sufficient length to be well entangled.

Consider two layers of polystyrene. Each layer consists of polystyrene chains that have been labelled with deuterium such that ~50% of the protons on the chains have been substituted with deuterium. One layer consists of a DHD-labelled polysty-

rene triblock copolymer where the lengths of the PSD endblocks are one-half that of the centre PSH block. The second layer is an HDH polystyrene triblock copolymer of equal molecular weight where the labelling has been reversed. HDH and DHD denote the sequencing of the normal polystyrene, PSH, and the perdeuterated polystyrene, PSD, blocks in the molecule. At temperatures above the glass-transition temperature of the polystyrene (~100 °C), the polymer chains begin to interdiffuse across the interface. If the motion of the polymer is the same for each portion of the molecule, the concentration of deuterium across the interface would remain constant. However, if the motion was curvilinear, that is biased towards reptation, this would no longer be true.

The experiment is shown schematically in Fig. 1. Here, the labelled polystyrene molecules are depicted by open and filled circles, where the open circles represent the deuterium-labelled portions of the molecule and the filled circles are the normal (protonated) portions of the chains. Initially, (Fig. 1a) the average concentration of the deuteriums-labelled portions of the molecules, ϕ_D , viewed normal to the interface is 0.5. As time proceeds, the molecules diffuse across the interface. If, as shown in Fig. 1b, the ends of the chains diffuse first across the interface, followed by the centres of the chains, then for times τ less than the reptation time τ_D (that is, the time it takes for a molecule to diffuse along its own contour), an excess of deuterium on the HDH side (and a depletion on the DHD side) will occur. This is shown in the concentration profile at the right of Fig. 1. As diffusion proceeds, the maximum and minimum in the profile will vanish and a constant concentration profile at 0.5 will again be found.

For these experiments, suitably labelled polystyrene chains must be prepared with well defined molecular weights and narrow molecular-weight distributions. The DHD polymer was synthesized by polymerizing deuterated styrene using a sec-butyl lithium initiator. Normal styrene was then added to the reaction mixture, and polymerization continued, forming a diblock copolymer. The diblock copolymer was then coupled using dichlorodimethylsilane to produce the desired triblock copolymer. This synthesis route ensures that the lengths of the end-labelled portions of the chains are equal. From size-exclusion chromatography the weight-average molecular weight, M_w , of the polymer was found to be 2.25×10^5 with a narrow molecular-weight distribution. An HDH polystyrene with a narrow molecular-weight distribution and $M_w = 2.45 \times 10^5$ was prepared

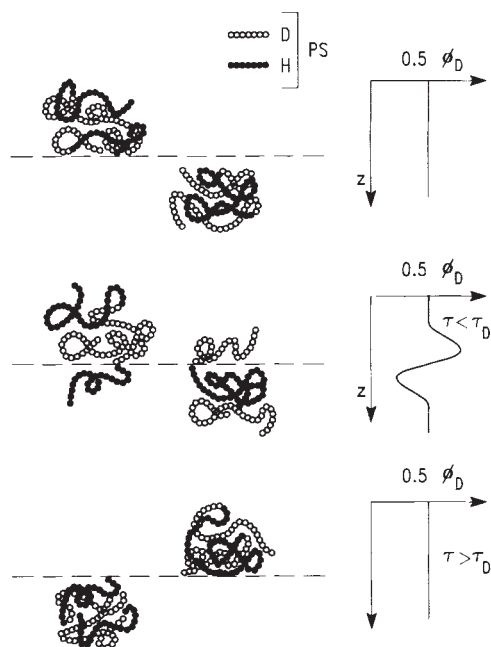


FIG. 1 Diagram of the interface between a bilayer of HDH- and DHD-labelled polystyrene (PS) showing only a few of the polymer chains. The open circles represent the portions of the chains labelled with deuterium and the filled circles are the protonated portions. a The initial interface; b, the interface after centre-of-mass diffusion over distances less than the radius of gyration; c the interface after the molecules have fully diffused across it. The graphs on the right depict the concentration of deuterium as a function of distance across the interface.

in a similar manner. The relative fractions of deuterium labelling were 0.57 and 0.5 for the HDH and DHD polymers, respectively. Films of the DHD polymer, ~100-nm thick, were prepared by spin-coating solutions of the DHD in toluene onto cleaned quartz substrates. These were annealed at 170 °C for 24 hr under vacuum to remove residual solvent and to allow the films to relax. A 100-nm film of the HDH polystyrene was then placed on top of the DHD layer as described elsewhere^{10,22}. In the whole process, well tested procedures assured the homogeneity of the films as well as a clean, sharp interface²³.

The profiles of the deuterium and hydrogen across the interface were determined by dynamic secondary-ion mass spectrometry (DSIMS) with a depth resolution of 5 nm. Using a Perkin Elmer 6300 secondary-ion mass spectrometer, a 2-keV O₂⁺ beam (200 nA directed 60° from the surface normal and rastered over an area of 500 µm × 500 µm) was used to sputter the polystyrene. Secondary ions of H⁺ and D⁺ were collected from the central 9% of the rastered area. A low-energy electron beam was used to compensate the positive surface charging that occurred. The depths of the sputtered craters were measured to convert the sputtering time to distance. The initial DSIMS profiles showed

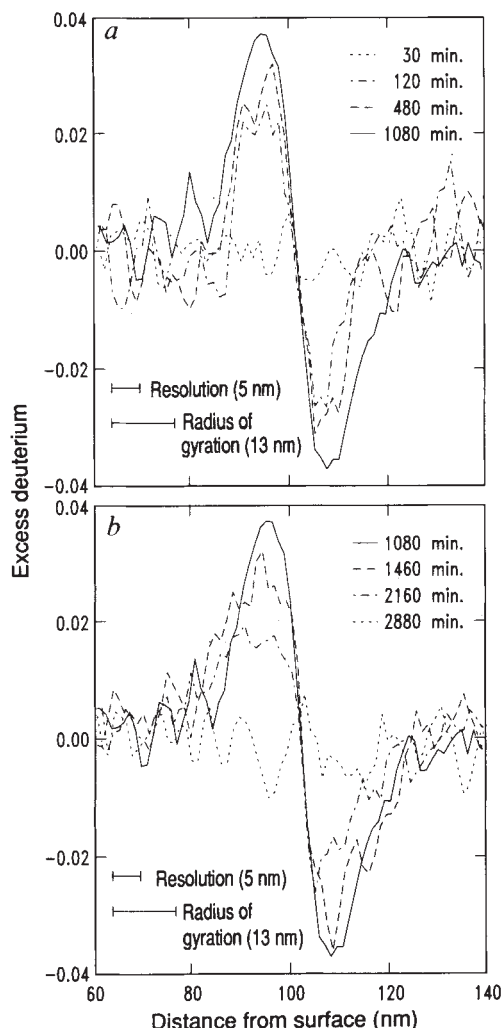


FIG. 2 Excess deuterium determined by DSIMS as a function of distance for the HDH- and DHD- polystyrene bilayers after interdiffusion at 118 °C for the times specified. In *a* the amplitude of the excess deuterium increases with time, whereas in *b* the excess deuterium decreases with time. *a*, ···, 30 min; ---, 120 min; - · -, 480 min; —, 1,080 min. *b*, —, 1,080 min; ---, 1,460 min; - · -, 2,160 min; ···, 2,880 min.

a difference between the H and D content in the HDH and DHD layers. These profiles were used as a baseline and subtracted from the remaining H and D depth profiles to yield the H and D excess. (For clarity, only the excess of D, normalized by the initial D signal in the DHD layer, is reported here.) The interdiffusion of the bilayers was performed by heating the samples in vacuum to 118 °C for the desired time, followed by quenching to room temperature. Figure 2*a* and *b* shows the excess of D as a function of depth for the different annealing times.

The rise and decay of the maximum and minimum in the profiles are clearly seen, which, taking into account the instrumental resolution, follow a behaviour characteristic of reptation. The results show that distances over which the maximum and minimum extend are comparable to the radius of gyration, R_g , of the polystyrene (indicated in Fig. 2). These results were checked against possible artefacts. First, we note that subtracting the initial profile from subsequent profiles is not correct. By simple fickian diffusion the interface will broaden to give a similar effect. However, the amplitudes of the signal from such an effect are too small to be observable over the timescale studied. Also, the maximum and minimum would be seen on the opposite sides of the interface as experimentally observed. Second, surface-segregation effects were examined on separate films and found to be of no consequence. The results were independent of the sequencing of the layers. Finally the experiment was designed to minimize the excess (non-ideal) free energy of mixing between the HDH and DHD layers, which acts to retard the diffusion.

These results demonstrate that the diffusion of polymer chains across the interface must occur by some kind of reptative process. Isotropic fluctuations of chain segments across the interface are not observable in this experiment because of the nature of the labelling. In fact, nearly 120 min (slightly less than the Rouse relaxation time of the entire chain, that is, the relaxation time of internal modes in the chain) were required before any excess was observable. The spatial separation of the maximum and minimum in the profile of D excess was time-invariant but the breadth of the signal increased with time. The amplitude of the excess reached a maximum at ~1,080 min and had vanished by 2,880 min, which corresponds well to the calculated reptation time τ_d for these polymers at 118 °C is ~2,200 min. Thus, it has been shown that the chain ends lead the chain centres over a time during which the whole chain has diffused a distance equal to R_g . This is curvilinear or reptative motion. Our unpublished simulations based on pure reptation yield results in agreement with the observations shown here. Similar studies are being done on different molecular-weight pairs of polystyrene, both above and below the entanglement molecular weight ($\sim 2 \times 10^4$). Experiments are also in progress using other techniques with better spatial resolution to provide a definitive set of data to test the details of theoretical predictions and computer simulations²⁴. □

Received 14 April; accepted 23 July 1993.

- de Gennes, P.-G. *J. Chem. Phys.* **55**, 572-579 (1971).
- Doi, M., Edwards, S. J. *J. chem. Soc., Faraday Trans. 2* **74**, 1789-1801, 1802-1817, 1818-1832 (1978).
- Klein, J. *Nature* **271**, 143-145 (1978).
- Green, P. F., Mills, P. J., Palmstrom, C., Mayer, J. W. & Kramer, F. J. *Phys. Rev. Lett.* **53**, 2145-2148 (1984).
- Richter, D. *Phys. Rev. Lett.* **64**, 1389-1392 (1990).
- Butera, R. et al. *Phys. Rev. Lett.* **66**, 2088-2091 (1991).
- Green, P. F., Monnerie, L. & Fetters, L. J. *Macromolecules* **21**, 2404-2412 (1988).
- Ylitalo, C. M., Fuller, G. G., Abetz, V., Stadler, R. & Pearson, D. S. *Rheol. Acta* **29**, 543-555 (1990).
- Lee, A. & Wool, R. P. *Macromolecules* **19**, 1063-1068 (1986); **20**, 1924-1927 (1987).
- Karim, A. thesis, Northwestern Univ. (1990).
- Whitlow, S. J. & Wool, R. P. *Macromolecules* **24**, 5926-5938 (1991).
- Osaki, K. et al. *Macromolecules* **22**, 2457-2460 (1989).
- Wool, R. P. *Structure and Strength of Polymer Interfaces* (Hanser, New York (1993)).
- Brochard-Wyart, F. & de Gennes, P. G. *Physico-Chemical Hydrodyn.* **4**, 313-322 (1983).
- Tirrell, M., Adolf, D. & Prager, S. *Springer Lecture Notes in Appl. Math.* **163**, 37-45 (Springer, Heidelberg, 1984).
- Harden, J. L. *J. Phys. Paris*, **51**, 1777-1784 (1990).

17. Reiter, G. & Steiner, U. *J. Phys. II* **1**, 659–671 (1991).
18. Kremer, K. & Grest, G. S. *J. chem. Phys.* **92**, 5057–5086 (1991).
19. Paul, W., Binder, K., Heermann, D. W. & Kremer, K. *J. Phys. II* **1**, 37–60 (1991).
20. Paul, W., Binder, K., Heermann, D. W. & Kremer, K. *J. chem. Phys.* **95**, 7726–7740 (1991).
21. Crabb, C. C. & Kovac, J. *Macromolecules* **18**, 1430–1435 (1985).
22. Russell, T. P., Karim, A., Mansour, A. & Felcher, G. P. *Macromolecules* **21**, 1890–1893 (1988).
23. Karim, A., Mansour, A., Felcher, G. P. & Russell, T. P. *Mater. Res. Soc. Symp.* **171**, 329–333 (Mater. Res. Soc., Pittsburgh, 1990).
24. Agrawal, G. et al. *Macromolecules* (submitted).

Dynamic enhancement of cation migration in a Zintl alloy by polyanion rotation

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ZINTL alloys are compounds of an alkali metal A with a polyvalent metal M in which transfer of an electron from A to M leads to bonding behaviour in the M^- ions typical of elements one column to the right in the Periodic Table. For example, ASn and APb contain tetrahedral M_4^{4-} polyanions in both the crystalline and liquid phases¹ whereas AAl alloys crystallize in tetrahedrally bonded networks typical of Group IV elements². These bonding patterns also determine the character of the disorder in the solid at high temperature. We have recently identified a plastic crystal phase in CsPb just below the melting point, in which the Pb_4^{4-} polyanions undergo jump reorientations³; LiAl, in contrast, is metallic, with mobile Li^+ ions⁴. Here we report unusual disordering behaviour in the Zintl alloy NaSn, in which both types of dynamic disorder appear: rapid reorientations of the Sn_4^{4-} polyanions enhance jump migration of the Na^+ cations by means of a ‘paddle-wheel’ effect. The two types of disorder are strongly coupled, so that only one disordering transition takes place as the melting point is approached.

NaSn is an alloy well known since 1928, when Hume-Rothery⁵ made a careful study of its phase diagram. Although Hume-Rothery’s diagram has since been modified in some details⁶, his general scheme has been validated by subsequent work; for example, his values for the transition and melting temperatures of the compound NaSn agree with our recent calorimetry measurements⁷ to within 1–2 °C. An interesting feature of the phase diagram is a transition (over the entire composition range of the solid) at 483 °C, ~95 °C below the congruent melting point. Hume-Rothery referred to this simply as a ‘polymorphic phase transformation in the solid’, and to our knowledge it has not been explored since.

The transition is dramatically reflected in both thermodynamic properties. Figure 1a shows the enthalpy of NaSn derived from our calorimetric measurement⁷. The entropy change at the transition, $T_1 = 484$ °C, is $\Delta S_1 = 4.95$ J mol⁻¹ K⁻¹, comparable to the entropy of melting, $\Delta S_m = 8.48$ J mol⁻¹ K⁻¹. The electrical conductivity measured for NaSn as a function of temperature⁸ is shown in Fig. 1b, and shows a sharp drop at the transition followed by a rapid rise towards the melting point. This general behaviour is qualitatively consistent with current understanding about the electronic structure of NaSn: band-structure calculations^{9,10} show the low-temperature solid phase to be a narrow-gap semiconductor with a direct band gap of ~0.3 eV.

The low-temperature (β) phase has the NaPb crystal structure found in all the ASn and APb alloys (A = Na, ... Cs)¹¹. Nearly perfectly tetrahedral Sn_4^{4-} anions lie on a body-centred tetra-

gonal lattice separated by Na^+ cations on two types of site, namely Na(1) sites forming tetrahedra concentric with, and oppositely directed to, the Sn_4^{4-} tetrahedra and Na(2) sites forming squares in the (a - b) plane, also concentric with the Sn_4^{4-} tetrahedra. All Na sites can be considered to be shared between two Sn_4^{4-} tetrahedra. No crystallographic data are available, to our knowledge, for the high-temperature (α) phase. Neutron diffraction studies of the liquid¹² indicate that the Sn_4^{4-} polyanions survive into the melt, although the ‘first sharp diffraction peak’ characteristic of intermediate-range order, which in this case would result from a random packing of the tetrahedra, is not as pronounced as in other ASn and APb alloys apart from NaPb (ref. 1).

The relatively large entropy change of the β - α transition suggested that the α -phase might be dynamically disordered. To investigate this possibility, quasielastic neutron scattering (QENS) measurements were carried out on the IRIS spectrometer at the ISIS pulsed spallation source¹³. The IRIS spectrometer incorporates a detector at high angle with which a diffraction pattern can be collected, making it possible to monitor closely the changes in state with temperature and also to derive structural information about the current phase of the sample. The diffraction patterns of the β -phase were consistent with the reported structure¹¹, and no additional Bragg peaks were observed. At the β - α transition, the Bragg peaks around a scattering vector $Q \sim 1$ Å⁻¹ were replaced by a set of lower-symmetry peaks, consistent with a triclinic structure. (D.L.P., M.L.S. and J. W. Richardson, unpublished data.) At higher Q the Bragg peaks were greatly attenuated and replaced by diffuse scattering, peaking around $Q \sim 2$ Å⁻¹, as in the liquid. All the Bragg peaks disappeared on melting and the remaining diffuse scattering was consistent with the reported liquid structure¹².

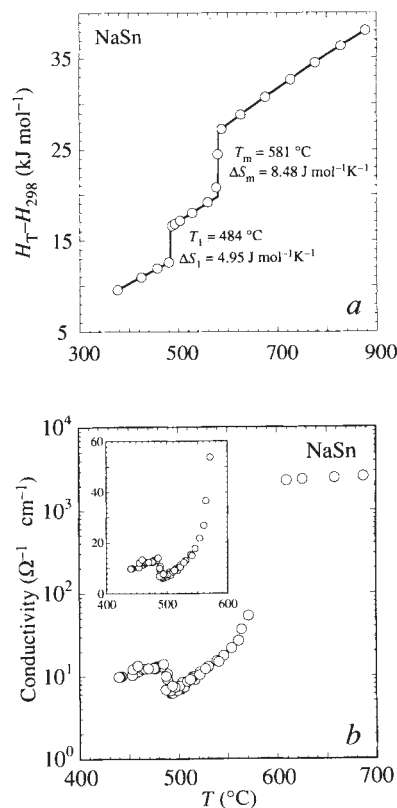


FIG. 1 a, Enthalpy, and b, electrical conductivity of NaSn as a function of temperature. The inset in b, shows the behaviour at the transition on an expanded scale (units on axes same as main graphs).

The QENS measurements showed no broadening of the elastic peak in the β -phase, the energy width remaining the same as for the room-temperature vanadium reference. At the β - α transition, dramatic quasielastic broadening was observed. After subtracting the container and furnace scattering, no significant elastic intensity was observed except at Q values near the Bragg peaks. Best estimates of intensities of elastic and quasielastic components at each Q were obtained by fitting delta and lorentzian functions with a maximum-entropy method¹⁴; after averaging over all Q values away from Bragg peaks, the elastic intensity was zero within experimental error. On melting, the quasielastic scattering broadened further. The intensities I_L and widths ΔE (full width at half-maximum) of the fitted lorentzian are shown as a function of Q in Fig. 2. The intensities are seen to rise to a maximum at $\sim 0.5\text{--}1\text{ \AA}^{-1}$ and then fall to a very low value at $1.8\text{ \AA}^{-1}$, after which they increase again. The widths behave similarly except they increase more sharply to a peak near $1.2\text{ \AA}^{-1}$ and the minimum is less deep.

Pre-melting of multistage melting in alloys can take various forms, ranging from massive production of positional defects to fast ion conduction¹⁵. Recently we identified an unusual two-stage melting process in the Zintl alloy CsPb by means of a rotationally disordered phase³. However, the Q dependence shown in Fig. 2b is more typical of fast ion conduction like that encountered in metal hydrides or oxides at high temperatures. A model often applied to incoherent scattering from such systems is that of Chudley and Elliott¹⁶, which predicts a behaviour of the form.

$$\Delta E = h/\pi\tau(1 - \sin(QL)/QL) \quad (1)$$

where τ is the characteristic jump time, L is the jump distance and h is Planck's constant. This function is plotted in Fig. 2b with $h/\pi\tau = 0.25\text{ meV}$ ($\tau = 5.3\text{ ps}$) and $L = 3.75\text{ \AA}$. The model function describes the data reasonably well up to the minimum at $Q \sim 1.7\text{ \AA}^{-1}$, and the value of L is similar to the closest Na-Na interatomic distances in the β -phase ($3.6\text{--}3.72\text{ \AA}$)¹¹. The

coherent scattering (51% of the total scattering in the case of Na) is harder to estimate. In the simplest case—a single type of atom site and uniform relaxation about occupied sites—the scattering is only weakly dependent on Q and the width follows equation (1) (ref. 17). In real fast ion conductors, for example fluorite structures¹⁸, the diffuse scattering intensity is concentrated at specific regions of the Brillouin zone, depending on the details of the structure and relaxation processes. In any case, equation (1) should give a reasonable approximation to the directionally averaged behaviour. Note that the lack of significant elastic scattering confirms the rapid mobility of the Na^+ ions, as the relatively large incoherent cross-section of Na would otherwise lead to appreciable elastic scattering.

Because the Na^+ ions are highly mobile, the Sn^{4-} ions must remain localized in order to preserve the rigidity of the solid. The disappearance of the Bragg peaks at higher Q , however, suggests either a massive Debye-Waller factor associated with large-amplitude Sn^{4-} vibrations or a form factor associated with rotational disorder of the rapid reorientations of Sn_4^{4-} polyanions. The former explanation is impossible, as the large attenuation of the Bragg peaks at Q as low as $2\text{ \AA}^{-1}$ implies a mean linear displacement of $0.75\text{ \AA}$, or 25% of the Sn-Sn distance, which is much larger than the Lindemann criterion for melting. Thus, the only possible interpretation involves rotational disorder of the Sn_4^{4-} polyanions, similar to that observed for Pb polyanions in CsPb (ref. 3). The disorder must be dynamic, because significant diffuse elastic scattering is observed. A model of jump reorientations between four unequally spaced orientations (similar to that used for CsPb), shows that the quasielastic scattering intensity associated with these is relatively low up to $Q \sim 1.7\text{ \AA}^{-1}$, after which it begins to rise rapidly. This model can therefore explain the rise in I_L and the increase in ΔE above the Chudley-Elliott model values at $Q \sim 1.8\text{ \AA}^{-1}$. In principle, such merohedral disorder (jumps between discrete orientations) gives rise to a small amount of diffuse coherent elastic scattering, which was identified in the CsPb data, whereas isotopic rotations give none¹⁹ (Sn has an extremely small incoherent cross-section, so incoherent elastic scattering from it is negligible). However, the diffuse coherent elastic scattering decreases with increasing rotational disorder, and the question of whether a small amount of elastic scattering is present is difficult to answer unambiguously, as it depends sensitively on the correction for the container and furnace scattering.

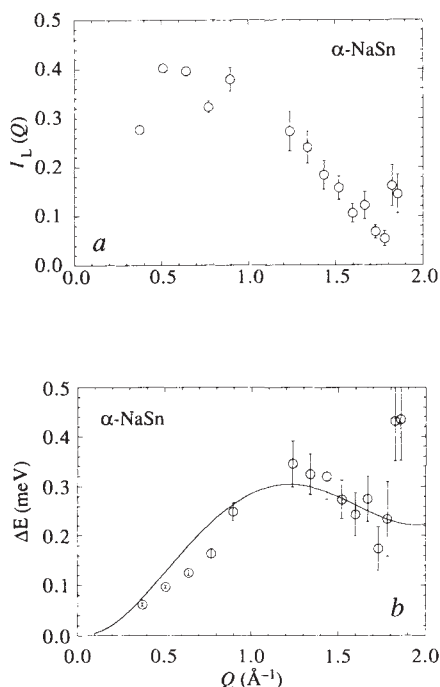


FIG. 2 a, Intensity, and b, full width at half-maximum of the lorentzian function fitted to quasi-elastic scattering from NaSn at 500 °C. The solid curve in b is calculated from a simple jump-diffusion model (equation (1) in the text).

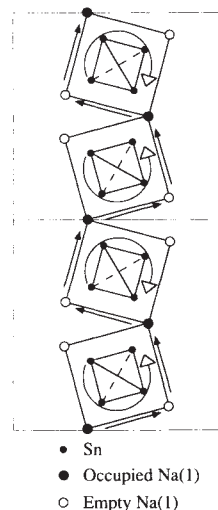


FIG. 3 Schematic illustration of 'paddle-wheel' migration in NaSn. The geometry shown is based on the a - b plane of the β -phase (body-centred tetragonal) crystal structure; the actual α -phase may have a triclinic distortion.

Thus, we have identified a disordered phase in NaSn in which dynamic translational disorder of the cations is enhanced by dynamic rotational disorder of the polyanions. The two processes must be strongly coupled as only one phase transition is observed before melting. A diagram of the motions involved is shown in Fig. 3. Furthermore, the time constants must be similar, as a single lorentzian function adequately fits the data. The 'paddle-wheel' migration of the Na^+ ion resembles that of Li^+ in $\alpha\text{-Li}_2\text{SO}_4$ (and some other sulphates), where the behaviour might be anticipated because of the high stability of the sulphate ion²⁰. Its occurrence in a semiconducting alloy such as NaSn is remarkable because the polyanions are not expected to be so stable at high temperatures. More recently, coupled rotational and translational disorder has been observed in AC_{60} alloys²¹. Further work is needed to establish the crystal symmetry and atomic structure of $\alpha\text{-NaSn}$, the nature of the rotational disorder, the geometry of the diffusive jumps and the relation of these microscopic properties to the changes in entropy and electrical conductivity (electronic and ionic) at the phase transition. □

Received 26 April; accepted 5 August 1993.

1. Reijers, H. T. J. *et al.* *Phys. Rev.* **B40**, 6018–6029 (1989); **41**, 5661–5666 (1990).
2. Zintl, E. & Brauer, G. *Phys. Chem.* **B20**, 245–249 (1933).

3. Price, D. L., Saboungi, M.-L., Reijers, H. T. J., Kearley, D. & White, R. *Phys. Rev. Lett.* **66**, 1894–1897 (1991); *Phys. Rev.* **B44**, 7289–7296 (1991).
4. Brun, T. O., Susman, S., Rowe, J. M. & Rush, J. J. *Solid St. Ionics* **5**, 417–419 (1981).
5. Hume-Rothery, W. J. *chem. Soc.* **131**, 947–963 (1928).
6. Müller, W. & Volk, K. Z. *Naturf.* **338**, 275–278 (1978).
7. Saboungi, M.-L., Johnson, G. K. & Price, D. L. in *Proc. NATO ASI on Statics and Dynamics of Alloy Phase Transformations*, Rhodes, June 22–July 3 (eds Gonis, A. & Turchi, P.) (Plenum, New York, in the press).
8. Saboungi, M.-L., Fortner, J., Richardson, J. W., Doyle, M. & Enderby, J. E. J. *Non-cryst. Solids* **156–158**, 356–361 (1993).
9. Springelkamp, E., de Groot, R. H., Geertsma, W., van der Lugt, W. & Mueller, F. M. *Phys. Rev.* **B32**, 2319–2325 (1985).
10. Meijer, J. A. thesis, Univ. Groningen (1988).
11. Müller, W. & Volk, K. Z. *Naturf.* **328**, 709–710 (1977).
12. Alblas, B. P., van der Lugt, W., Dijkstra, J., Geertsma, W. & van Dijk, C. J. *Phys.* **F13**, 2465–2477 (1983).
13. Boland, B. C. Report No. RAL-90-041 (Rutherford-Appleton Laboratory, Chilton, 1990).
14. Sivia, D. S. & Volk, K. J. *chem. Phys.* **96**, 170–178 (1992).
15. Ubbelohde, A. R. *The Molten State of Matter: Melting and Crystal Structure* (Wiley, Chichester, 1978).
16. Chudley, C. T. & Elliott, R. J. *Proc. Phys. Soc.* **77**, 353–361 (1961).
17. Anderson, N. H., Clausen, K. N. & Kjems, J. K. in *Neutron Scattering, Methods of Experimental Physics* Vol. 23 (eds Price, D. L. & Sköld, K.) Part B, 187–241 (Academic, San Diego, 1987).
18. Hutchings, M. T. *et al.* *J. Phys.* **C17**, 3903–3940 (1984).
19. Neumann, D. A. *et al.* *Phys. Rev. Lett.* **67**, 3808–3811 (1991).
20. Börjesson, L. & Torrell, L. M. *Phys. Rev.* **B32**, 2471–2477 (1985).
21. Zhu, Q. *Bull. Am. Phys. Soc.* **38**, 426–427 (1993).

ACKNOWLEDGEMENTS. This work was supported by the US Department of Energy. We thank J. E. Enderby for many discussions, C. J. Carlile and D. S. Sivia for guidance in the data analysis, R. L. Hitterman for preparing Fig. 3 and the staff at ISIS for making these measurements possible.

Non-aqueous synthesis of giant crystals of zeolites and molecular sieves

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IN the rapidly developing area of advanced zeolite materials science, the development of novel optical, electronic and magnetic materials^{1–5} requires the synthesis of large single crystals of a well defined habit. Crystals of a specified size and shape are needed to fabricate membranes and sensor devices^{6,7}. Large crystals of zeolites and molecular sieves are essential for refining structures and elucidating the intrinsic absorption and diffusion properties of these materials^{8,9}. Here we present a method for growing uniformly large and morphologically well defined single crystals of all-silica, aluminosilicate and aluminophosphate molecular sieves with dimensions in the size range 0.4–5.0 mm. Our synthetic method relies on the use of a non-aqueous solvent, a mineralizer, an optional organic templating species and reagent amounts of water. We have prepared both large-pore and small-pore materials of various framework composition and structure type.

Natural zeolites can occur as large single crystals. These are, however, often impure and/or rare, and require growth conditions that cannot be reproduced in the laboratory¹⁰. Large zeolite crystals are difficult to produce in the laboratory by conventional hydrothermal synthesis, where crystal formation is controlled to a large extent by the solubility of the reagent gel particles, the rate of generation of nucleation centres and the solubility of the resulting products¹¹. Usually, the low solubility of the crystalline products leads to a high degree of supersaturation, causing high nucleation rates that result in many small crystals. In a few successful cases, careful control of parameters such as the composition of the synthesis mixture, dilution, temperature, agitation and crystallization time have led to large-crystal materials for specific zeolite syntheses^{12–16}. The use of slow-release com-

TABLE 1 Synthesis of large zeolite crystals

Zeolite	Reagents* (Molar ratios)	Temp. (°C)	Time (days)
Dodecasil-3C	1.5SiO ₂ :2HF/py:6H ₂ O :16py	200	7
Ferrierite	1.5SiO ₂ :2HF/py:8H ₂ O :4PrNH ₂ :16py	180	7
Aluminosilicate Ferrierite	0.125Al ₂ O ₃ :2SiO ₂ :2HF/Et ₃ N:0.5TPA-Br :12Et ₃ N	180	12
Silicalite	2SiO ₂ :2HF/Et ₃ N:6H ₂ O 2PrNH ₂ :0.5TPA-Br :12Et ₃ H	180	12
3(C ₂ H ₅) ₃ NH(Al ₃ P ₄ O ₁₆)	1Al ₂ O ₃ :1.6P ₂ O ₅ :5.9Et ₃ N :14 polyethylene glycol	195	10

* Abbreviations for reagents: py, pyridine; PrNH₂, propylamine; Et₃N, triethylamine; TPA-Br, tetrapropylammonium bromide.

plexing agents, seeding, sub- and supragravity conditions to increase crystal size have met with limited success^{17–19}.

Our research efforts have focused on non-aqueous solvent systems. Successful large-crystal syntheses have never been performed in pyridine, triethylamine, tetraethylene glycol, diethylene glycol, tetrahydrofuran, dimethylformamide, dimethylsulphoxide, sulfolane, lutidine and mineral oil²⁰. Although previous work has established the synthesis of a variety of molecular sieves (including aluminophosphate-structure types, pentasils, sodalite and Ba-T (barium-containing zeolite T) from non-aqueous systems including glycols, alcohols and pyridine), these studies have been unsuccessful for growing large single crystals^{21–24}.

Our synthesis systems include, in combination with the non-aqueous solvent, a mineralizing agent, reagent quantities of water, an optional organic template, silica or silica and alumina, or alumina and phosphorous oxide sources. The reagents used were fumed silica (Cab-O-Sil M-5 or EH-5; Aerosil 200, Cabot), pseudoboehmite alumina (Dispal 180 or 23N4-80, Vista), phosphoric acid (85%, Aldrich), hydrogen fluoride-pyridine (70% (by weight) HF in pyridine, Aldrich), triethylamine trihydrofluoride (98%, Aldrich), propylamine, tetrapropylammonium bromide (98%, Aldrich), pyridine, triethylamine, polyethylene glycol (average molecular weight 200, Aldrich or BDH) and, if necessary, other organic additives from Aldrich or BDH.

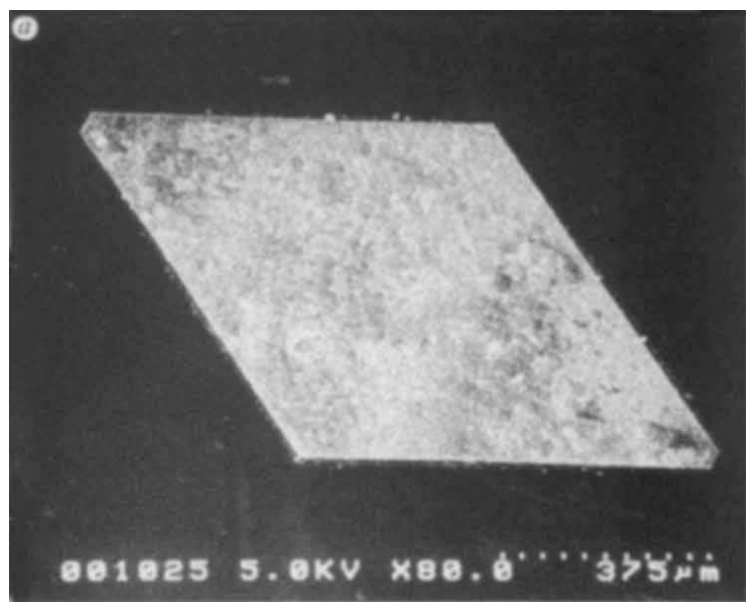
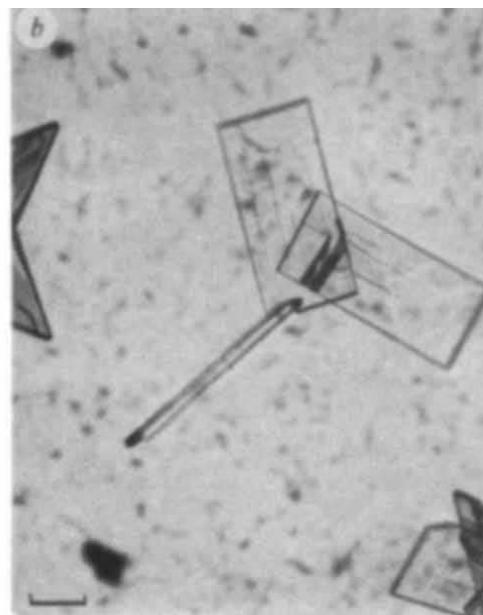


FIG. 1 a, Scanning electron micrograph of an aluminosilicate ferrierite crystal. Note that the texture of the crystal surfaces seen in the SEM originates mainly from the coating procedures; dotted scale bar =



375 µm. b, Optical photograph of all-silica ferrierite crystals calcined at 900°C; scale bar = 100 µm.

In the case of all-silica and aluminosilicate syntheses, reaction mixtures were prepared by first combining organic solvent, water and propylamine, if used. Then the required amount of a fluoride source was added to the solution, followed by a preweighed amount of silica or a mixture of silica and alumina. If necessary, tetrapropylammonium bromide was added, with agitation, to

the resulting mixture. In the case of the aluminophosphate synthesis, a slurry of alumina in polyethylene glycol was prepared initially followed by the slow addition of phosphoric acid and then triethylamine. All the final mixtures were stirred to homogeneity. The reactions were performed in Teflon-lined stainless steel autoclaves (capacity 45 ml) at 120–200 °C. The autoclaves

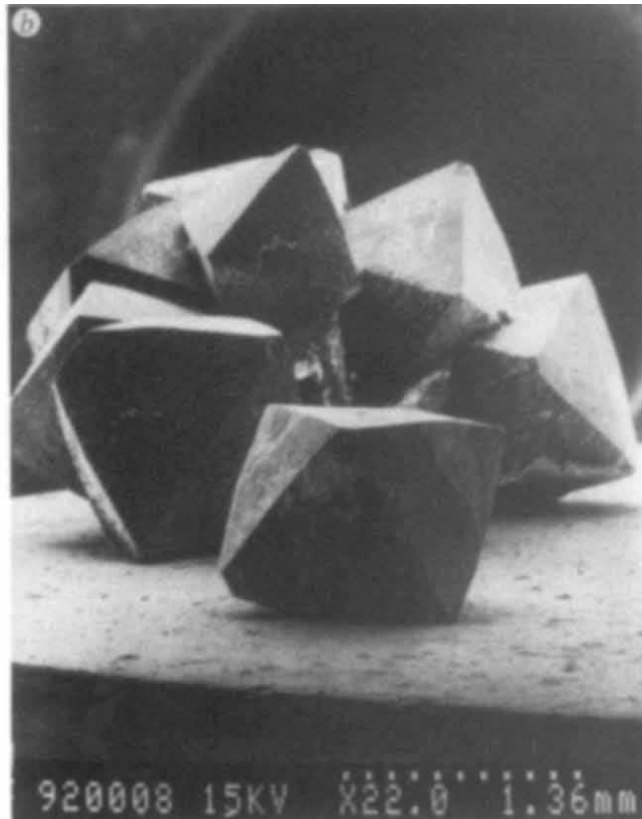
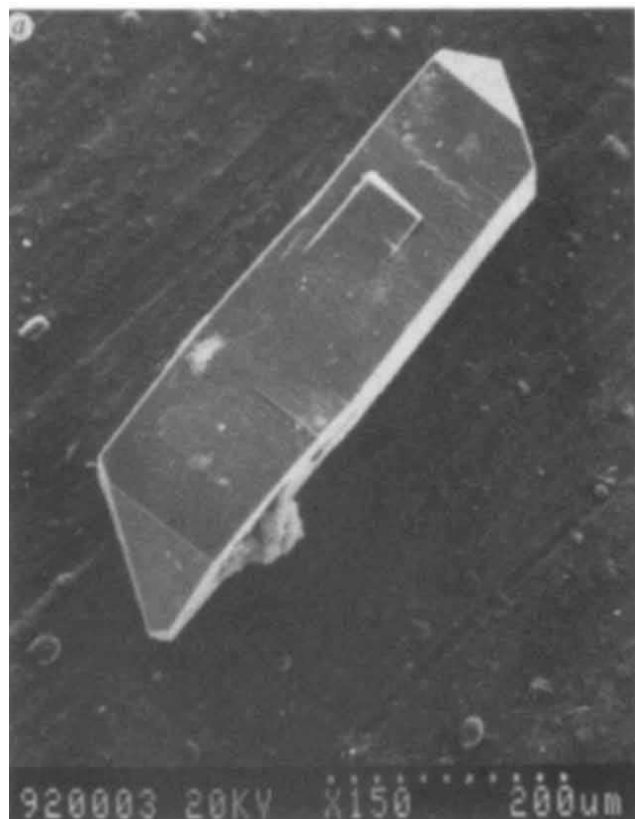


FIG. 2 Scanning electron micrographs of crystals of silicalite-1 (a) and dodecasil-3C (b). Dotted scale bar in a = 200 µm, in b = 1.36 mm.

were neither shaken nor stirred during the heating period. To recover products, autoclaves were removed from the oven and quenched with cold water. Most of the large-crystal products were obtained with a good yield and found to be of uniform size (within a given batch) and of well defined morphology. Molar compositions of typical reaction mixtures and synthesis conditions for the preparations of large crystals are listed in Table 1.

The nature of the synthesis products was found to depend on, among other variables, the basicity of the organic solvent, the choice of template and HF concentration. Hydrogen fluoride, which itself is highly associated in the liquid phase by means of hydrogen bonding, gives remarkably stable polyhydrogen fluorides²⁵ when reacted with pyridine and alkylamines.

The 70% (by weight) HF in pyridine solution studied in detail²⁵ was found to be pyridinium polyhydrogen fluoride containing a small amount of free HF in equilibrium, thereby acting as a reservoir of anhydrous HF in a selected organic solvent. Thus HF-pyridine and HF-alkylamines are novel mineralizers that allow one to control the amount of water present in the reaction medium. A key point of this study is that in the non-aqueous systems water is used as a reactant that can be added as required to dissolve precursor species. The basicity of the pyridine/ F^-/OH^- system seems to be much less than aqueous systems due to ion-pair formation. The organic components, together with the hydroxide and fluoride ions, act in unison to narrow the range and control the release and solubility of reactive solution species. Supersaturation levels and nucleation rates are lowered in these organothermal systems relative to the hydrothermal ones.

Examination of the crystalline products under a polarizing microscope showed no evidence of twinning. Images of representative crystals of these materials are displayed in Figs 1–3. Note the millimetre dimensions and well-defined morphologies. Crystal structures and phase purity of all the materials studied have been determined using single-crystal and powder X-ray diffraction (XRD) techniques, respectively. In the case of all-silica ferrierite, scanning electron microscopy revealed a uniform collection of purely silicious crystals with a plate-like morphology. Powder XRD showed that the sample was single phase, and a single-crystal XRD study determined a *Pmnn* orthorhombic unit cell with $a=18.807$ Å, $b=14.094$ Å, $c=7.432$ Å and the composition $Si_{36}O_{72} \cdot 2(C_5H_5N) \cdot 2HF$. The resultant large crystals

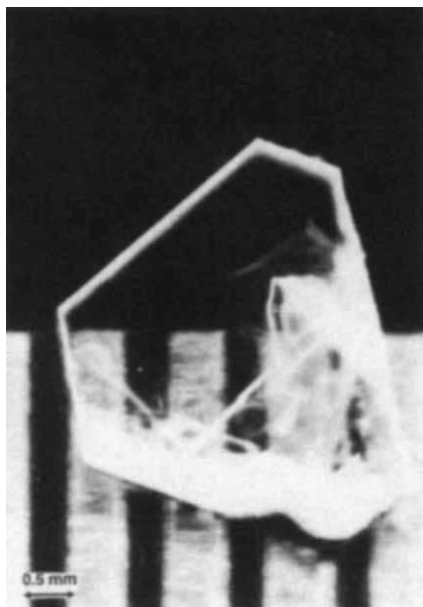


FIG. 3 Optical photograph of a crystal of a novel microporous aluminophosphate $3(C_2H_5)_3NH(Al_3P_4O_{16})$.

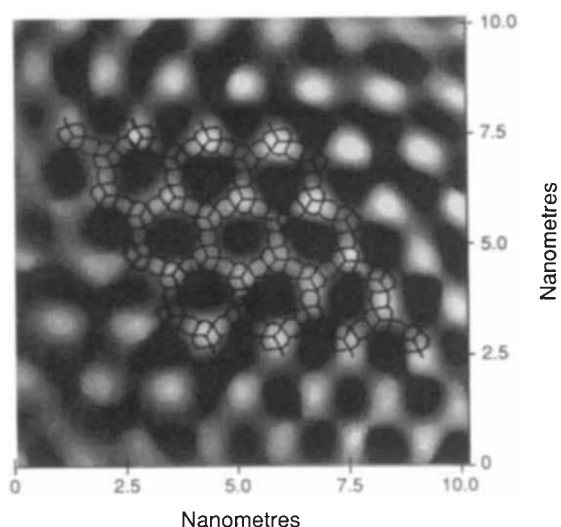


FIG. 4 Atomic force microscopy image of the surface of the $3(C_2H_5)_3NH(Al_3P_4O_{16})$ crystal superimposed on the [001] crystallographic projection of the structure.

tals of aluminium-containing ferrierite exhibited a plate-like morphology similar to the all-silica species. Energy-dispersive X-ray analysis (EDX) in the scanning electron microscope performed at various spots across polished crystals revealed a homogeneous Al distribution with $Si/Al=17$. The structure of this material was refined in the *Immm* orthorhombic space group with unit-cell parameters $a=18.901$ Å, $b=14.108$ Å, $c=7.434$ Å, showing the expected increase in the unit-cell dimensions compared with the all-silica analogue. Based on this refinement and the EDX analytical data, the unit-cell composition of the Al-containing ferrierite crystal was established to be $Al_2Si_{34}O_{72} \cdot 2(C_5H_5N) \cdot 2HF$. The single-crystal XRD indicated that the pyridine was located in the 8-ring channels of the structure, while the HF was found to be disordered in the main 10-ring pore. It was established that the occluded species can be removed from the structure by calcination in air at $750^\circ C$ without any observable deterioration of the crystal quality. N_2 adsorption measurements on the calcined crystals yielded a surface area of $286 \text{ m}^2 \text{ g}^{-1}$ and a porosity value of 0.129 ml g^{-1} , which agree well with values expected for a very pure ferrierite.

Several other molecular sieves were also synthesized in large crystal forms including silicalite-1, dodecasil 3-C and a novel microporous aluminophosphate. Single crystal studies of these materials established a *Pnma* orthorhombic unit cell with $a=20.026$ Å, $b=19.936$ Å, $c=13.391$ Å and a composition of $Si_{96}O_{192}$ for silicalite-1, a *I42d* tetragonal unit cell with $a=13.644$ Å, $c=19.520$ Å and a composition of $Si_{17}O_{34} \cdot (C_5H_5N)$ for dodecasil-3C and a *P3* trigonal unit cell with $a=13.092$ Å, $c=10.093$ Å and a composition of $((C_2H_5)_3NH)_3Al_3P_4O_{16}$ for the novel aluminophosphate. The latter is structurally similar to the recently reported $(NH_3(CH_2)_4NH_3)_{1.5}Al_3P_4O_{16}$ material²⁶, which has been synthesized as large crystals by using polyethylene glycol solvent. It is built of $AlPO_4$ -5-like sheets ([001] projection), but with phosphate caps over each six-membered ring. The high degree of surface perfection can be appreciated from the close correspondence between the [001] crystallographic projection of the structure and the atomic force microscopy image, as shown in Fig. 4. □

Received 10 February; accepted 23 July 1993.

- Ozin, G. A., Kuperman, A. & Stein, A. *Angew. Chem., int. Edn engl.* **28**, 359–376 (1989).
- Stucky, G. D. & MacDougall, J. E. *Science* **247**, 669–678 (1989).
- Cox, S. D., Gier, T. E., Stucky, G. D. & Bierlein, J. J. *Am. chem. Soc.* **110**, 2986–2987 (1988).

4. Caro, J. et al. *Adv. Mater.* **4**, 273–276 (1992).
5. Ozin, G. A. *Adv. Mater.* **10**, 612–649 (1992).
6. Geus, E. R., Schoonman, J. & Van Bekkum, H. in *Mater. Res. Soc. Symp. Proc.* **233**, 231–236 (1991).
7. Yan, Y. & Bein, T. *Mater. Res. Soc. Symp. Proc.* **233**, 175–180 (1991).
8. Beshman, K., Kokotailo, G. T. & Reikert, L. *Stud. Surf. Sci. Catal.* **39**, 355–365 (1988).
9. Muller, U. & Unger, K. K. *Stud. Surf. Sci. Catal.* **39**, 101–108 (1988).
10. Hay, R. L. in *Natural Zeolites* (eds Sand, L. B. & Mumpton, F. A.) 135–143 (Pergamon, Oxford, 1978).
11. Zhdanov, S. P. *Adv. Chem. Ser.* **101**, 20–43 (1971).
12. Guth, J. L. et al. *Am. chem. Soc. Symp. Ser.* **398**, 176–195 (1989).
13. Guth, J. L., Kessler, H. & Wey, R. *Stud. Surf. Sci. Catal.* **28**, 121–128 (1986).
14. Muller, U., Brenner, A., Reich, A. & Unger, K. K. *Am. chem. Soc. Symp. Ser.* **398**, 346–359 (1989).
15. Chae, H. K. et al. *Am. chem. Soc. Symp. Ser.* **455**, 528–540 (1991).
16. Bogomolov, V. N. & Petranovsky, V. P. *Zeolites* **6**, 418–419 (1986).
17. Hayhurst, D. T., Melling, P. J., Kim, W. J. & Bibbey, W. *Am. chem. Soc. Symp. Ser.* **398**, 233–243 (1989).
18. Scott, G., Thompson, R. W., Dixon, A. G. & Sacco, A. *Zeolites* **10**, 44–50 (1990).
19. Sano, T. et al. *Zeolites* **12**, 801–805 (1992).
20. Ozin, G. A., Kuperman, A. & Nadimi, S. US Patent (filed 1991).
21. Bibby, D. M. & Dale, M. P. *Nature* **317**, 157–158 (1985).
22. van Erp, W. A., Kouwenhoven, H. W. & Nanne, J. M. *Zeolites* **7**, 286–288 (1987).
23. Qisheng, H., Shouhua, F. & Ruren, X. *J. chem. Soc., chem. Commun.* 1486–1487 (1988).
24. Qisheng, H. & Ruren, X. *J. chem. Soc., chem. Commun.* 783–784 (1990).
25. Olah, G., Nojima, M. & Kerbs, I. *Synthesis* 779–780 (1973).
26. Thomas, J. M. et al. *J. chem. Soc., chem. Commun.* 929–931 (1992).

ACKNOWLEDGEMENTS. We thank the Dow Chemical Company for financial support and A. Lough, P. Rudolf, N. Coombs and P. Enzel for technical assistance.

Anthropogenic lead in Antarctic sea water

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ANTARCTICA is believed to be a relatively pristine continent, mainly because of its remote location and the atmospheric circulation patterns that limit the transport of industrial aerosols into the Antarctic polar cell^{1–4}. This perception is apparently supported by the extremely low concentrations of lead in Antarctic surface waters—an observation that has been interpreted as showing insignificant contamination by anthropogenic lead⁵. The isotopic composition of lead in other natural waters has been used as a tracer of the sources of lead, and in particular to identify anthropogenic inputs^{6,7}. Here we apply this approach to Antarctic surface waters, and show that despite the low concentrations of lead in these waters (which are confirmed by our measurements), their isotopic composition reveals a significant contribution of lead from industrial sources. The extremely low concentrations of lead in these waters appear to be due to biological scavenging of the lead during periods of intense primary production.

Surface-water samples (unfiltered) were collected on the AMERIEZ (Antarctic Marine Research at the Ice Edge Zone) and RACER (Research in Antarctic Coastal Ecosystems Rates and Processes) expeditions in 1987 and 1988 (Fig. 1). The AMERIEZ collections extended over mesoscale frontal systems along the marginal ice zone in the Weddell and Scotia Seas during periods of intense ($0.2\text{--}0.5\text{ g C m}^{-2}\text{ d}^{-1}$) biological productivity⁸. The RACER collections were obtained during similarly productive periods along the Antarctic Peninsula. All the samples were collected, stored, processed and analysed with rigorous trace-metal-clean techniques⁹. Lead concentrations and isotopic compositions were measured by thermal ionization mass spectrometry using established methods, following a dithizone extraction^{6,7}. The isotopic composition measurements were calibrated with concurrent measurements of NIST SRM 981 (com-

mon lead isotopic composition standard). The concentration measurements were verified by concurrent analyses of seawater standards of the National Research Council of Canada (CASSI and NASSI); they were also intercalibrated by replicate analyses of lead and iron, using graphite furnace atomic absorption spectrometry with established methods, following an APDC/DDDC extraction¹⁰.

The wide range in seawater lead concentrations from 9.6 to $103 \times 10^{-12}\text{ M}$ (pM) (Table 1) is attributed to (1) the small amounts of dissolved lead in Antarctic surface waters and (2) the relatively large amounts of particulate lead in some of those waters. The former was demonstrated recently by measurements of low dissolved-lead concentrations ($4\text{--}30\text{ pM}$) in Antarctic surface waters⁵, and was consistent with measurements of low lead concentrations (11 pM) in Antarctic rainwater¹¹. The latter was shown by the positive correlation ($r=0.893$, simple linear regression) between lead and iron concentrations, which ranged from 0.12 to $191 \times 10^{-9}\text{ M}$ (nM), in the unfiltered seawater samples. Because dissolved iron concentrations in Weddell Sea surface waters are $\leq 1\text{ nM}$ ^{12,13}, the high concentrations of lead in the unfiltered sea water must have been associated with particulates. In spite of those particulates, some of the total-lead concentrations in Antarctic surface waters were still lower than the dissolved-lead concentrations in any other oceanic surface waters⁶.

These analyses confirmed a projection that total lead concentrations in the Antarctic could be $\leq 10\text{ pM}$, based on parallels between atmospheric inputs and surface-water concentrations of lead in other oceanic surface waters¹⁴. The projection was derived from the aeolian lead flux in the Antarctic polar cell ($100\text{ fmol cm}^{-2}\text{ yr}^{-1}$, where $\text{fmol}=10^{-15}\text{ mol}$) which is at least two orders of magnitude lower than aeolian lead fluxes in the Arctic polar cell ($14\text{ pmol cm}^{-2}\text{ yr}^{-1}$), North Pacific Easterlies ($34\text{ pmol cm}^{-2}\text{ yr}^{-1}$), North Pacific Westerlies ($72\text{ pmol cm}^{-2}\text{ yr}^{-1}$), North Atlantic Westerlies ($820\text{ pmol cm}^{-2}\text{ yr}^{-1}$), South Pacific Easterlies ($10\text{ pmol cm}^{-2}\text{ yr}^{-1}$) and South Pacific Westerlies ($48\text{ pmol cm}^{-2}\text{ yr}^{-1}$). The projection assumed that lead concentrations in the Antarctic are limited by particulate scavenging processes during periods of intense primary productivity in the austral summer. The confirmation of this projection substantiates the proposal that low lead concentrations in Antarctic surface waters reflect both the relative isolation of this area from both natural and anthropogenic inputs of lead, and the efficacy of scavenging processes associated with seasonally robust primary productivity.

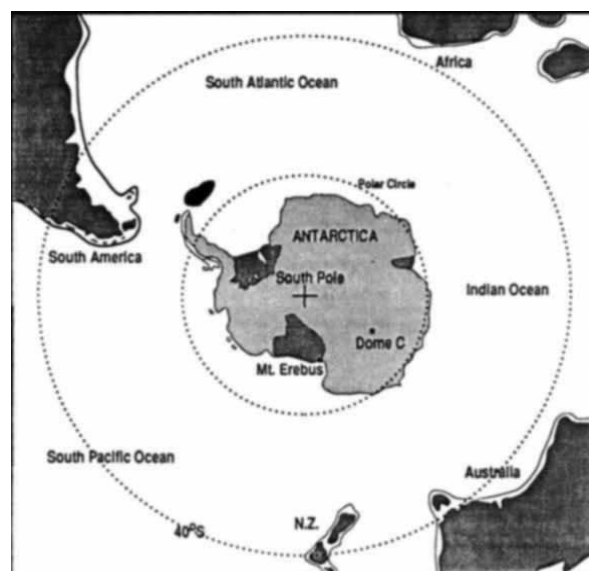


FIG. 1 Sample locations (solid areas) in the Antarctic Ocean.

TABLE 1 Lead concentrations and isotopic compositions in Antarctic seawater

Sample date Lat., long.	[Pb] (pM)	$^{206}\text{Pb}/^{204}\text{Pb}$	$^{207}\text{Pb}/^{204}\text{Pb}$	$^{208}\text{Pb}/^{204}\text{Pb}$
5-3-87 62°34' S, 61°27' W	12.4	17.957 ± 0.016	15.554 ± 0.020	37.648 ± 0.060
26-3-87 63°25' S, 60°09' W	82.3	17.816 ± 0.016	15.559 ± 0.019	37.445 ± 0.062
28-6-87 62°50' S, 62°12' W	—	17.614 ± 0.015	15.480 ± 0.021	37.043 ± 0.068
13-6-88 61°17' S, 42°16' W	20.5	17.444 ± 0.018	15.497 ± 0.023	37.104 ± 0.051
14-6-88 61°22' S, 41°54' W	28.6	17.057 ± 0.016	15.519 ± 0.012	36.814 ± 0.030
25-6-88 59°52' S, 47°52' W	9.6	18.708 ± 0.015	15.651 ± 0.014	38.337 ± 0.043
26-6-88 60°21' S, 47°53' W	—	18.896 ± 0.018	15.676 ± 0.016	38.511 ± 0.045
24-7-88 57°42' S, 35°25' W	71.5	18.245 ± 0.008	15.615 ± 0.007	37.946 ± 0.018
27-7-88 58°31' S, 40°00' W	37.1	18.078 ± 0.009	15.583 ± 0.008	37.866 ± 0.018
28-7-88 59°47' S, 39°59' W	—	18.152 ± 0.012	15.578 ± 0.010	37.888 ± 0.018
4-8-88 59°23' S, 43°58' W	16.1	17.863 ± 0.017	15.568 ± 0.015	37.643 ± 0.045
8-8-88 58°01' S, 44°48' W	—	17.436 ± 0.012	15.545 ± 0.008	37.187 ± 0.024
9-8-88 57°57' S, 48°00' W	—	18.055 ± 0.015	15.582 ± 0.019	37.761 ± 0.062
13-8-88 59°04' S, 48°01' W	12.8	18.433 ± 0.017	15.600 ± 0.017	38.064 ± 0.046
	13.1	—	—	—
	—	17.769 ± 0.016	15.561 ± 0.019	37.471 ± 0.062
	103	17.972 ± 0.020	15.573 ± 0.019	37.726 ± 0.062
	29.8	18.352 ± 0.020	15.573 ± 0.015	37.991 ± 0.040
	31.1	18.373 ± 0.020	15.596 ± 0.020	38.081 ± 0.040

Values are for unfiltered Antarctic surface waters. (Data from replicate collections from the same area are listed separately.) Uncertainties in isotope ratios are $\pm 95\%$ confidence limit.

Isotopic compositions of the sea water (Table 1) fit simple linear regressions ($r \geq 0.928$), which show that there are two primary sources of lead in Antarctic surface waters (Fig. 2). The non-radiogenic component is attributed to industrial lead emissions in the Southern Hemisphere. The more radiogenic component is not ascribed to industrial lead emissions from the Northern Hemisphere, because the interhemispheric transport of lead in both the troposphere and ocean surface currents is minimal⁶. As previously noted, the lead concentrations in Antarctic surface waters were also positively correlated with iron concentrations, which are not measurably increased in oceanic waters by anthropogenic inputs. Therefore, the other component of lead in Antarctic surface waters appears to be derived from natural sources.

The anthropogenic component of lead in Antarctic surface waters is characteristic of atmospheric emissions from the combustion of gasoline (with lead additives) in the Southern Hemisphere. This characterization is based on the contemporary (1985–1988) isotopic composition of urban aerosols in Perth, Australia ($1.057 \leq ^{206}\text{Pb}/^{207}\text{Pb} \leq 1.062$) (K. Rosman, personal communication). The major sources of this lead are the extremely old (1.5 Gyr) Broken Hill and Mount Isa ore deposits in Australia ($^{206}\text{Pb}/^{207}\text{Pb} \sim 1.04$), with relatively smaller amounts from the Mississippi Valley deposits in North America ($^{206}\text{Pb}/^{207}\text{Pb} \sim 1.35$) (K. Rosman, personal communication). Lead from the Australian deposits have previously been identified as the principal components of lead in gasoline and urban aerosols in Australia¹⁵, urban aerosols and individuals in South Africa¹⁶, aerosols in the South Pacific¹¹, and surface waters in the South Pacific⁶. Overall, measurements of the isotopic composition of lead in the Antarctic, South Pacific, Africa and Australia attest to the pandemic dispersion of industrial lead aerosols emitted in the Southern Hemisphere; this is consistent with the global increase of industrial lead emissions, which are orders of magnitude above natural lead emissions¹⁷.

We suggest that Antarctic surface waters contain comparable amounts of natural and industrial lead. The natural lead may be derived from several sources: volcanic emissions such as those from Mount Erebus in Antarctica; aeolian dust from South America; and sediment transport by ice rafting. Mount Erebus (77° 32' S, 167° 10' E) is the largest (3,974 m high), most active volcano in Antarctica, with an anorthoclase phonolite lava lake that continuously emits gases and aerosols into the atmosphere¹⁸. These emissions have been traced in ambient air to a distance of 1,000 km from their origins on Ross Island¹⁹. The isotopic compositions of leads from the Erebus volcanic province²⁰ are close to the radiogenic component defined by the lead in Antarctic surface waters. But isotopic composition of volcanic rocks in Marie Byrd land, as well as lavas and sediments of adjacent land masses in the Southern Ocean (for example, South Sandwich Island)²¹ and Chile²², are also close to the radiogenic end-member. In addition, the dust in Dome C ice during the last glacial maximum was found to be derived from South America, based on neodymium and strontium isotopic compositions²³. Relatively large amounts of particulate lead could also be advected into the Antarctic by coastal polynyas, based on the transport of sediment-laden ice (30–600 mg l⁻¹) over 100-km seaward of the continental shelf in the Arctic²⁴. Contributions from each of those sources are presumably reflected in the isotopic compositions of pelagic sediments in the Southern Ocean^{21,25}, which are in turn similar to the isotopic composition of the radiogenic component in Antarctic sea water.

The relative amount of anthropogenic lead in Antarctic surface waters was estimated to range from 30 to 70%, based on the isotopic composition of Mount Erebus volcanics, and from 0 to 60%, based on the isotopic composition of a South American crustal source²². In both cases, the relative proportions of natural and anthropogenic lead in Antarctic surface waters varies considerably at different sites. The higher proportions of natural lead in many of the seawater samples contrasts with the low proportion (<10%) of crustal and volcanic lead in atmospheric

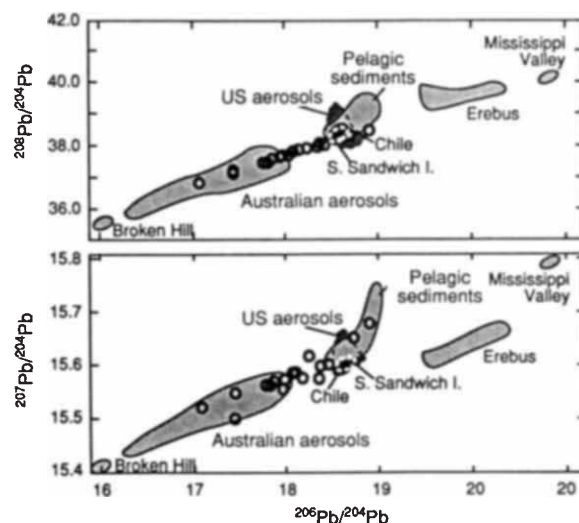


FIG. 2 Lead isotopic compositions of Antarctic surface waters (○) collected during the AMIRIEZ and RACER expeditions in 1987 and 1988. The seawater lead appears to be composed of industrial lead ores from the Broken Hill and Mississippi Valley deposits (which were the primary components of contemporary Australian and US urban aerosols), and natural leads from adjacent terrestrial sources. The latter may include volcanic emissions, aeolian dust and weathered sediments advected into the Antarctic by coastal polynyas. The isotopic compositions of these natural lead sources are consistent with the isotopic compositions of pelagic sediments in the Antarctic, which are also similar to the radiogenic lead in the overlying waters.

aerosols collected from the Antarctic Peninsula, as estimated by Dick²⁶. Although these mass-flux estimates have not been corroborated by isotopic composition analyses, they indicate that the amount of natural lead is relatively greater in Antarctic surface waters than in the overlying atmosphere. This is consistent with our proposal that lead in Antarctic surface waters is derived primarily from industrial lead aerosols, which are soluble, and natural lead particulates, whose residence time in the atmosphere and surface waters is relatively short and whose distribution is highly variable. The variability in lead concentrations and isotopic compositions may also reflect point source inputs of industrial lead from ships, aircraft, and research operations in Antarctica.

In summary, isotopic compositions of Antarctic surface waters demonstrate that anthropogenic inputs of lead extend to the most remote region of the Earth. The contamination occurs in spite of a strong circumpolar atmospheric convergence barrier that restricts the transport of industrial lead aerosols into the Antarctic polar cell¹⁻⁴. This atmospheric barrier contributes to the low total-lead concentrations in some Antarctic surface waters, which are lower than the dissolved-lead concentrations of any other oceanic surface waters. It also makes small inputs of lead from natural sources relatively substantial. Such small inputs may include volcanic emissions, aeolian depositions of dust and the ice-rafting of sediments by coastal polynyas. However, both anthropogenic and natural inputs of lead to Antarctic

surface waters appear to be scavenged rapidly during periods of intense primary productivity, which we believe accounts for the exceptionally low concentrations of dissolved lead in Antarctic surface waters. □

Received 17 February; accepted 6 August 1993.

1. Boulton, C. F. & Patterson, C. C. *J. geophys. Res.* **92**, 8454–8465 (1987).
2. Peel, D. A. *Nature* **323**, 200 (1986).
3. Shaw, G. E. *Rev. Geophys.* **26**, 89–112 (1988).
4. Wolff, E. *Antarctic Sci.* **2**, 189–205 (1990).
5. Westerlund, S. & Öhman, P. *Geochim. cosmochim. Acta* **55**, 2127–2146 (1991).
6. Flegel, A. R. & Patterson, C. C. *Earth planet. Sci. Lett.* **64**, 19–32 (1983).
7. Flegel, A. R., Nriagu, J. O., Niemeyer, S. & Coale, K. H. *Nature* **339**, 455–458 (1989).
8. Smith, W. O. & Garrison, D. L. *Oceanogr.* **3**, 22–29 (1990).
9. Patterson, C. C. & Settle, D. M. *Nat. Bur. Stand. Spec. Publ.* **422**, 321–351 (1976).
10. Bruland, K. W., Coale, K. H. & Mart, L. *Mar. Chem.* **17**, 285–300 (1985).
11. Patterson, C. C. & Settle, D. M. *Mar. Chem.* **22**, 137–162 (1987).
12. Martin, J. H., Gordon, R. M. & Fitzwater, S. E. *Nature* **345**, 156–158 (1990).
13. Westerlund, S. & Öhman, P. *Mar. Chem.* **35**, 199–217 (1991).
14. Patterson, C. C., Boulton, C. Flegel, A. R. *Geophys. Monogr.* **33**, 101–104 (1985).
15. Guison, B. L., Tiller, K. G., Mizon, K. J. & Merry, R. H. *Envir. Sci. Technol.* **15**, 691–696 (1981).
16. Manton, W. I. *Arch. envir. Health* **32**, 149–159 (1977).
17. Nriagu, J. O. & Pacyna, J. M. *Nature* **333**, 134–139 (1988).
18. Kyle, P. R. in *Volcanoes of the Antarctic Plate and Southern Oceans, Antarctic Research Series* **48**, 18–25 (Am. Geophys. Un., Washington DC, 1990).
19. Chuan, R. L., Palais, J., Rose, W. I. & Kyle, P. R. *J. Atmos. Chem.* **4**, 467–477 (1986).
20. Sun, S. S. & Hanson, G. N. *Contr. Miner. Petrol.* **52**, 77–106 (1975).
21. Barreiro, B. *Geochim. cosmochim. Acta* **47**, 817–822 (1983).
22. Hildreth, W. & Moorbath, S. *Contr. Miner. Petrol.* **98**, 455–489 (1988).
23. Grousset, F. E. et al. *Earth planet. Sci. Lett.* **111**, 175–182 (1992).
24. Reimnitz, E., McCormick, M., McDougall, K. & Brouser, E. *Arct. Alp. Res.* **25**, 83–98 (1993).
25. Chow, T. J. & Patterson, C. C. *Geochim. cosmochim. Acta* **26**, 263–308 (1962).
26. Dick, A. L. *Geochim. cosmochim. Acta* **55**, 1827–1836 (1991).

Effect of pasture age on soil trace-gas emissions from a deforested area of Costa Rica

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MEASUREMENTS of the flux of nitrous oxide—an important greenhouse gas—from recently formed pasture in the Amazon basin have shown a threefold increase relative to the flux from the original forest soil¹. Based on these measurements, Luizao *et al.*¹ estimated that present rates of conversion from forest to pasture supply up to 1 Tg of N₂O–N to the atmosphere each year, corresponding to ≤25% of the current imbalance between sources and sinks of this gas. But this estimate assumes that such conversion produces elevated fluxes that remain constant in time. To assess the validity of this assumption, we present measurements of trace-gas fluxes from Costa Rican pastures of varying ages. Nitrogen oxide fluxes peak during the first ten years after conversion, but decline thereafter to values that are even lower than the original forest fluxes. We conclude that previous studies have overestimated the contribution of pastures to the global budget of nitrous oxide, and that accurate predictions of soil–atmosphere trace-gas fluxes will require a detailed knowledge not only of current land use, but also of land-use history.

Soil emissions are the largest known source of atmospheric N₂O (refs 2, 3). The soils of tropical forests alone may emit 3–6 Tg N₂O–N (1 Tg = 10¹² g) of the total annual production of 14 Tg N₂O–N (refs 4, 5). Emissions of nitric oxide (NO) from the soil of tropical moist forests are of similar magnitude to N₂O

emissions^{6,7}. Nitric oxide is a critical precursor to the formation of ozone in the troposphere. Ozone is a greenhouse gas and a strong oxidant. The combustion of fossil fuels and biomass is the main source of atmospheric NO (ref. 8). In rural areas, however, soil emissions of NO may equal or exceed combustion

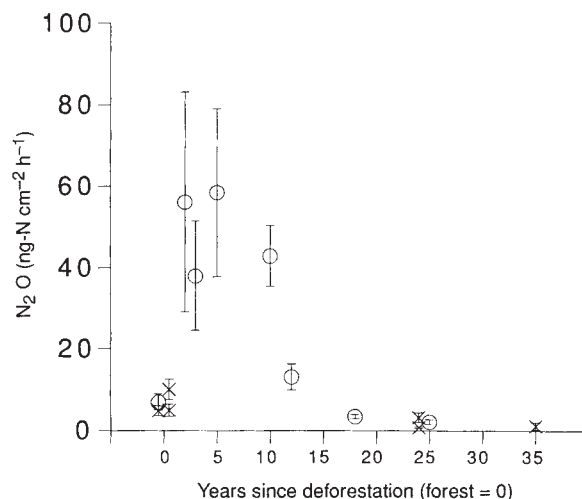


FIG. 1 Average soil emissions (± 1 standard error) of N₂O plotted against pasture age (time since deforestation) for La Selva (x) and Guácimo (O). Forest sites are shown at 0 yr. Significant differences were found among site means (analysis of variance, $P < 0.01$). Five 20-ml samples for N₂O and CH₄ were removed by a nylon syringe at regular intervals during 28-min assays of vented chambers²⁵. Samples were analysed using electron capture and flame ionization gas chromatography with electronic integration of peak area^{14,26}. Sample peak areas were compared with commercially prepared standard mixtures of N₂O and CH₄ which are calibrated at the NOAA Climate Monitoring and Diagnostics Laboratory, Boulder, Colorado. Fluxes were calculated using the linear regression of concentration against time.

TABLE 1 Soil densities and soil-atmosphere CH₄ fluxes

Site age (yr)	Bulk density 0–5 cm depth (g cm ⁻³)	CH ₄ flux	
		Dry months (mg CH ₄ m ⁻² d ⁻¹)	Wet months
Guácimo area			
Forest	0.67 (0.06)	−1.98 (1.14)	−0.66 (0.64)
2	0.78 (0.04)	2.20 (8.05)	2.52 (0.68)
3	0.83 (0.05)	−0.77 (1.50)	−0.06 (0.67)
5	0.82 (0.04)	−0.80 (0.82)	2.58 (4.85)
10	0.84 (0.03)	−1.87 (0.65)	2.99 (6.48)
12	0.80 (0.04)	−0.75 (2.41)	1.53 (0.65)
18	0.98 (0.07)	−1.37 (0.30)	1.35 (3.72)
25	0.79 (0.05)	−1.29 (0.61)	0.18 (1.43)
La Selva area			
Forest	0.63 (0.05)	−1.89 (0.27)	−0.86 (0.31)
Forest	0.58 (0.05)	−1.90 (0.59)	−0.97 (0.49)
Forest	0.66 (0.08)	−2.01 (0.12)	−0.95 (0.78)
24	0.71 (0.04)	−0.65 (0.52)	0.89 (3.12)
24	0.78 (0.06)	−0.94 (0.67)	1.48 (3.34)
35	0.71 (0.08)	−0.70 (0.48)	−0.27 (0.90)

Average soil bulk densities and soil-atmosphere CH₄ fluxes by site (value in parentheses is standard deviation). Positive values of methane flux indicate a net transfer of methane from the soil to the atmosphere (emission) whereas negative values indicate net transfer of methane from the atmosphere to the soil (consumption). Soil bulk densities were determined for 8–23 replicates at each site using 100-ml stainless steel rings.

sources⁹. The effect of conversion of moist and wet tropical forests on soil NO emissions is unknown.

Well-drained soils generally act as sinks for atmospheric methane (CH₄). Soils consume about 40 Tg CH₄ yr⁻¹, equivalent to the current annual rate of increase in CH₄ concentration and about 10% of the total emissions, 400–500 Tg CH₄ yr⁻¹ (refs 10–12). Cultivation and nitrogen fertilization can diminish the consumption of methane by soil^{13–15}.

We sampled the soil-atmosphere fluxes of N₂O, NO and CH₄ from forest sites and derived pastures in two areas of the Atlantic lowlands of Costa Rica. The first area, near the La Selva Biological Station (10° 26' N, 84° W), included three replicates each of old-growth forest and pasture. Each replicate was sampled monthly from October 1990 to September 1991 (January to December 1991 for NO). The actively grazed pastures were cleared from forest 24–35 yr before our study. The second area, located near Guácimo (10° 12' N, 83° 32' W), included one forest site and seven active pastures that had been cleared 2–25 yr before sampling. The Guácimo sites were sampled eight times during the period February to November 1992. Eight measurements of N₂O+CH₄ flux and four measurements of NO flux were made per month at each site. Sampling dates were chosen randomly from a pool of available dates each month to avoid a bias from changing weather. All cleared sites had been immediately set to pasture after forest clearance except for an 18-yr-old site which was briefly ploughed and cultivated. In each area we located sites on a single soil unit, each classified as a Humitropept²⁸.

Both the average annual N₂O emission from forest soil at La Selva and the 8-month averages from the forest site near Guácimo (5–10 ng N₂O-N cm⁻² h⁻¹) (Fig. 1) are similar to short-term measurements made in the same region¹⁶. Emissions of N₂O from young pastures (2–10 yr) exceed forest soil emissions by a factor of 5–8. This result is consistent with a threefold increase in N₂O emission measured at 3-yr-old pasture versus forest on an oxisol near Manaus, Brazil¹. In contrast, older active pastures (>12 yr) at both La Selva and Guácimo emitted significantly less (one-half to one-third as much) N₂O than forest soils.

The pattern of emissions for NO also depends on pasture age (Fig. 2). Young pasture soils emit far more NO than older pasture soils. In contrast to N₂O, NO emissions from young (<12 yr) pasture soils at the Guácimo sites were not significantly different from the forest soil emissions. In both areas, the annual average forest soil NO flux still exceeds the flux from old pastures (>12 yr). Although the magnitude of soil NO emissions for forests and young pastures is similar, we would expect greater escape of NO to the atmosphere from the pastures because as much as 80% of the NO released from soil may be consumed by reaction and deposition in the dense forest canopy^{6,7,17}.

Forest soils consumed atmospheric CH₄ at rates equal to or greater than rates found at other moist and wet tropical forest sites (Table 1)^{5,14,18}. Dry-season CH₄ consumption in forest soils exceeded wet-season consumption by a factor of 2–3, which is consistent with the finding that soil methane consumption is limited by gaseous diffusion^{10,19,20}. Pasture sites consumed less methane than forest sites during the dry season. During the wet season, most pasture soils emitted methane to the atmosphere. Soil bulk density increases rapidly following conversion to pasture (Table 1). Compaction leads to poor drainage and reduced rates of gaseous diffusion, factors which limit aerobic methane consumption and promote anaerobic methane production. By calculating weighted averages of CH₄ flux (8 months wet and 4 months dry) for all sites, we estimate that the conversion of forest to cattle pasture in this region transforms a net sink for atmospheric CH₄ of -446 mg CH₄ m⁻² yr⁻¹ into a net source of +236 mg CH₄ m⁻² yr⁻¹.

The pattern of nitrogen oxide emissions that varies with pasture age may be understood with reference to predictable ecosys-

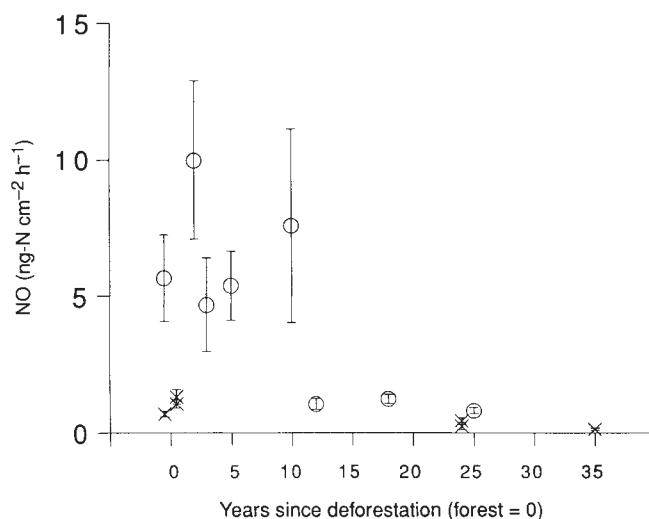


FIG. 2 Average soil emissions (± 1 s.e.) of NO plotted against pasture age for La Selva (x) and Cuácimo (o). Forest sites are shown at 0 yr. Significant differences were found among site means for the Guácimo area (analysis of variance, $P < 0.01$). The flux of nitric oxide was determined using methods similar to those used at tropical forest sites^{7,27}. We used dynamic chambers made from Teflon-coated aluminium. An air flow of 200–300 ml min⁻¹ was drawn through the chambers and analysed in the field after conversion of NO to NO₂ on a CrO₃ catalyst. Nitrogen dioxide was measured by a luminol-based chemiluminescence detector (Scintrex, LMA-3). An air-flow of 1,200–1,300 ml min⁻¹ was added to the chamber flow for optimum detector performance. A standard addition of NO in oxygen-free nitrogen (1 part per million (by volume) NO at 3 ml min⁻¹) was used to assure that the instrument operated in its linear range (1–50 parts per billion). Higher standard flow rates were used between each sample to quantify the instrument response. Fluxes were calculated from the initial slope of the trace of concentration against time (acquired on a battery-operated chart recorder).

tem properties. N_2O and NO are produced in soils by the processes of nitrification and denitrification^{21,22}. During the first decade following forest clearing, forest-derived organic matter at our sites decomposes rapidly²⁰. Mineralization of soil organic matter leads to high levels of nitrogen loss, as has been observed previously in our study region²³. Mineralized organic nitrogen inherited from the forest exceeds the capacity of pasture plants and microbes to sequester nitrogen. Thus, excess inorganic nitrogen becomes available to nitrifiers and denitrifiers that produce nitrogen oxides²⁴. The fraction of the available nitrogen converted to N_2O and NO depends on soil conditions^{21,22}. Soil compaction under pasture leads to poor drainage which favours the activity of denitrifiers. After about a decade of large nitrogen exports, the pastures become nitrogen-poor, so that nitrifier and denitrifier activity is limited and nitrogen oxide emissions fall below the original forest levels.

Very large increases (a factor of 5–8) of soil N_2O emissions are observed in young pastures compared with forests. Previous estimates (based on a tripling of forest emissions following conversion to pasture) have concluded that globally this source may exceed 1 Tg N_2O -N per yr. Although our results show that young tropical pastures are important N_2O emitters, we find that the period of high emissions is limited to only about a decade following forest clearing. Thereafter, N_2O emissions from pastures fall below forest levels, probably as a result of the depletion of available nitrogen. Therefore, we believe that the previous budget estimate of 1 Tg N_2O -N yr⁻¹ is best regarded as an upper bound. Rates of trace gas emissions from pastures formed following deforestation in the wet tropics vary according to the time since deforestation. Trace-gas budgets that consider only actual land use without considering the history of land use are likely to be inaccurate. □

Received 16 March; accepted 16 July 1993.

1. Luizão, F., Matson, P., Livingston, G., Luizão, R. & Vitousek, P. *Globi biogeochem. Cycles* **3**, 281–285 (1989).
2. Watson, R. T., Meira-Filho, L. G., Sanhueza, E. & Janetos, A. in *Climate Change 1992: The Supplementary Report to the IPCC Scientific Assessment* (eds Houghton, J. T., Callander, B. A. & Varney, S. K.) 23–46 (Cambridge Univ. Press, 1992).
3. Khalil, M. A. K. & Rasmussen, R. A. *J. geophys. Res.* **97**, 14651–14660 (1992).
4. Matson, P. A. & Vitousek, P. M. *Bioscience* **40**, 667–672 (1990).
5. Keller, M., Kaplan, W. A. & Wofsy, S. C. *J. geophys. Res.* **91**, 11791–11802 (1986).
6. Kaplan, W. A., Wofsy, S. C., Keller, M. & da Costa, J. M. *J. geophys. Res.* **93**, 1389–1395 (1988).
7. Bakwin, P. S. et al. *J. geophys. Res.* **95**, 16755–16763 (1990).
8. Logan, J. A. *J. geophys. Res.* **88**, 10785–10807 (1983).
9. Williams, E. J., Guenther, A. & Fehsenfeld, F. C. *J. geophys. Res.* **97**, 7511–7519 (1992).
10. Born, M., Dörr, H. & Levin, I. *Tellus* **42**, 2–8 (1990).
11. Cicerone, R. J. & Oremland, R. S. *Globi biogeochem. Cycles* **2**, 299–327 (1988).
12. Vaghjiani, G. L. & Ravishankara, A. R. *Nature* **350**, 406–409 (1991).
13. Steudler, P. A., Bowden, R. D., Melillo, J. M. & Aber, J. D. *Nature* **341**, 314–316 (1989).
14. Keller, M., Mitre, M. E. & Stailard, R. F. *Globi biogeochem. Cycles* **4**, 21–27 (1990).
15. Mosier, A., Schimel, D., Valentine, D., Bronson, K. & Parton, W. *Nature* **350**, 330–332 (1991).
16. Matson, P. A. & Vitousek, P. M. *Globi biogeochem. Cycles* **1**, 163–170 (1987).
17. Jacob, D. J. & Wofsy, S. C. *J. geophys. Res.* **95**, 16737–16754 (1990).
18. Delmas, R. A., Servant, J., Tathy, J. P., Cros, M. & Labat, M. *J. geophys. Res.* **97**, 6169–6179 (1992).

19. Dörr, H., Katruff, L. & Levin, I. *Chemosphere* **26**, 697–713 (1993).
20. Striegl, R. F. *Chemosphere* **26**, 715–720 (1993).
21. Firestone, M. K. & Davidson, E. A. in *Exchange of Trace Gases between Terrestrial Ecosystems and the Atmosphere* (eds Andreae, M. O. & Schimel, D. S.) 7–21 (J. Wiley, New York, 1989).
22. Conrad, R. in *Denitrification in Soil and Sediment* (eds Revsbech, N. P. & Sørensen, J.) 105–128 (Plenum, New York, 1990).
23. Matson, P. A., Vitousek, P. M., Ewel, J. J., Mazzarino, M. J. & Robertson, G. P. *Ecology* **68**, 491–502 (1987).
24. Robertson, G. P. & Tiedje, J. M. *Nature* **336**, 756–759 (1988).
25. Hutchinson, G. L. & Mosier, A. R. *Soil Sci. Soc. Am. J.* **45**, 311–316 (1981).
26. Butler, J. H., Elkins, J. W., Thompson, T. M. & Egan, K. B. *J. geophys. Res.* **94**, 14865–14877 (1989).
27. Davidson, E. A. et al. *J. geophys. Res.* **96**, 15439–15445 (1991).
28. Reiners, W. A., Bouwman, A. F., Parsons, W. F. J. & Keller, M. *Ecological Applications* (in the press).
29. Veidkamp, E. *Soil Sci. Am. J.* (in the press).

ACKNOWLEDGEMENTS. We thank M. Mitre, M. Nuñez, R. Vargas and D. Villegas for assistance in the field, and D. Schimel for comments. This work was supported by the Ecosystems Studies Program of the National Science Foundation, the US Environmental Protection Agency, and the Netherlands Foundation for the Advancement of Tropical Research (WOTRO). M.K. held a postdoctoral fellowship with the National Center for Atmospheric Research.

An Early Jurassic caecilian with limbs

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CAECILIANS are elongate, limbless, mostly fossorial amphibians. Less well known than frogs and salamanders, they are represented today by about 34 genera and 162 species¹. Until recently, the fossil record of caecilians consisted of only two vertebrae, one from the Palaeocene of Brazil², the other from the Late Cretaceous of Bolivia³. We report here the discovery of an extensive series of Early Jurassic caecilians which extends the fossil record of the group and reveals numerous features, including limbs, that are unknown among modern species. Although the new taxon possesses a tentacular fossa for a chemosensory organ and specializations of the jaw apparatus that are uniquely caecilian, other derived features are shared with salamanders and, among extinct Palaeozoic forms, with microsaurs. The configuration of the skull roof, which differs from the fenestrated condition typical of frogs, salamanders and putatively primitive living caecilians, is evidence of substantial evolutionary divergence between caecilians and other modern amphibian groups.

The new taxon is represented by 38 specimens, including 29 complete or fragmentary skulls and/or jaws, many with associated postcrania.

Order Gymnophiona Rafinesque, 1814
Family Eocaeciliidae, new
Eocaecilia micropodia gen. et sp. nov.

Etymology. Greek, *Eo*, dawn: the dawn caecilian; *mikros*, small; *pous*, foot: in reference to the diminutive limbs.

Holotype. Museum of Northern Arizona (MNA) V8066 (Fig. 1a, b).

Locality. Gold Spring, Adeii Eechii Cliffs, Coconino County, Arizona, USA (35° 45' 35" N, 111° 04' 51" W).

Horizon. Silty facies of the Kayenta Formation.

Age. Early Jurassic.

Referred specimens. MNA V8062, V8070; Museum of Comparative Zoology (MCZ) 9011A, 9169A, 9171A, 9231A, 9238A.

Diagnosis. A combination of derived and primitive characters. Derived characters shared with other gymnophiones: a tentacular fossa along the anterior orbital margin; exoccipitals, otic capsules and parasphenoid fused (= os basale); two upper rows of pedicellate teeth; a large retroarticular process on the lower jaw; an internal process on the pseudoangular; the pseudoangular and pseudodentary joined along an oblique suture. Primitive tetrapod characters: a complete complement of circumorbital bones (prefrontal, lacrimal, jugal, postorbital, postfrontal) and a

FIG. 1 Stereoscopic photographs. *a*, Dorsal and *b*, ventral views of the skull of the type specimen of *Eocaecilia micropodia* (MNA V8066; $\times 3.56$), gen. et sp. nov. *c*, Mesiodistal and *d*, labial views of a tooth crown (MCZ 9011A; $\times 108.5$) associated with a skull of *E. micropodia*. *e*, Dorsal view of a right retroarticular process and partially damaged stapes (MNA V8070; $\times 3.65$) that have become separated post mortem to reveal the apposing articular facets (arrows) of the joint between these two bones. *f*, First cervical vertebra (MCZ 9231A; $\times 8.3$) of *E. micropodia* in right lateral view; an arrow indicates the interglenoid tubercle. A foramen for a spinal nerve occurs at the junction of the pedicle and body. *g*, An articulated series of dorsal vertebrae of *E. micropodia* (MCZ 9169A; $\times 5.6$) with associated scapulocoracoid (Sc), humerus (H) and ulna. Arrows (l) indicate intercentra; these small ossicles, located between the ventral margins of adjoining vertebral bodies, articulate with the rib heads. *h*, Dorsal view of a left femur of *E. micropodia* (MNA V8062; $\times 5.8$). The bulbous femoral head is roughly oval in outline. The long axis of the head is oriented primarily dorsoventrally but is slightly tilted such that the dorsal half is more anteriorly situated than the ventral half. On both the medial and lateral sides of the head are shallow depressions, comparable to the foveae in some salamanders, that represent the attachment of acetabular ligaments from the pubis and ilium. A prominent, triangular trochanter is situated on the medioventral side of the proximal diaphysis. The configuration of the femoral head, the foveae and the trochanter are similar to the condition typical of modern salamanders (compare with *i*, *Salamandra salamandra*, MCZ 8045; $\times 1.95$).

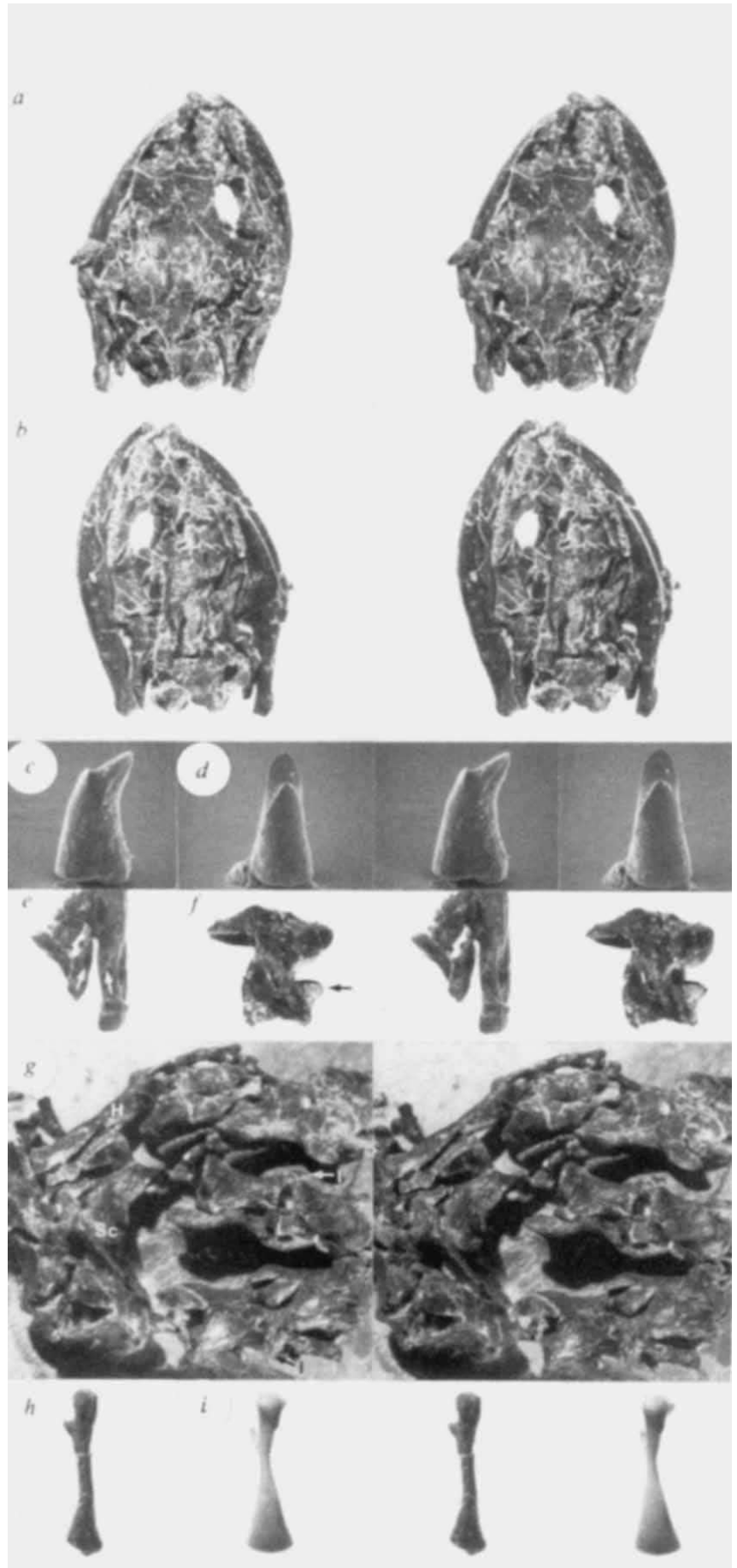


TABLE 1 Cranial, vertebral and appendicular proportions in various caecilians, salamanders and Palaeozoic amphibians

		Length (mm) ($\pm$ s.d.)		Ratio limb length:vertebral centrum length			
	N	Skull	Vertebral centrum	Humerus	Ulna	Femur	Tibia
Caecilians							
<i>Eocaecilia micropodia</i>		12.6	2.2	2.0	1.0	2.0	1.0
<i>Dermophis mexicanus</i>	5	15.9	3.4				
<i>Gymnopsis multiplicata</i>	1	19.3	4.0				
<i>Shistometopum thomensis</i>	2	7.9 $\pm$ 0.6	1.7 $\pm$ 0.4				
<i>Caecilia thomsoni</i>	1	6.8	1.8				
Palaeozoic amphibians							
<i>Proterogyrinus scheelei</i>		155	15.3	4.9	2.5	4.4	2.5
<i>Rhynchonkos stovalli</i>	1	18.3	2.4	3.4		3.9	
<i>Microbrachis pelikani</i>	6	16.7 $\pm$ 3.9	2.1 $\pm$ 0.5	5.2		2.8	
<i>Asaphestra intermedia</i>	2	34.3 $\pm$ 5.9	4.7 $\pm$ 1.4	4.5		4.6	
Salamanders							
<i>Necturus maculosus</i>	6	32.1 $\pm$ 4	7.0 $\pm$ 1.0	1.6	0.9	1.7	0.8
<i>Cryptobranchus alleganiensis</i>	11	45.3 $\pm$ 5	10.9 $\pm$ 1.0	1.8	0.9	1.9	1.0
<i>Desmognathus</i> spp.	10	12.9 $\pm$ 2	2.9 $\pm$ 0.5	2.0	1.0	2.3	1.3
<i>Pseudotriton montanus</i>	6	12.3 $\pm$ 1	3.1 $\pm$ 0.4	2.1	1.1	2.2	1.2
<i>Plethodon jordani</i>	3	10.0 $\pm$ 0.5	2.0 $\pm$ 0.2	2.9	1.8	3.2	1.8
<i>Plethodon</i> spp.	2	12.5 $\pm$ 1	2.7 $\pm$ 0.2	2.5	1.5	2.8	1.6
<i>Ambystoma maculatum</i>	12	13.5 $\pm$ 1	3.7 $\pm$ 0.5	2.3	1.4	2.3	1.3
<i>Salamandra salamandra</i>	11	14.2 $\pm$ 2	3.6 $\pm$ 1.0	2.8	1.6	2.7	1.5
<i>Taricha granulosa</i>	12	14.3 $\pm$ 1	3.9 $\pm$ 0.2	2.9	1.7	2.6	1.4

Relative lengths of limb bones are expressed in terms of maximal lengths of vertebral centra, rather than skull or body lengths which are more variable. All data are from specimens in the Museum of Comparative Zoology except *Dermophis mexicanus*⁸ (M. H. Wake, personal communication), *Proterogyrinus scheelei*¹⁶, *Rhynchonkos stovalli*, *Microbrachis pelikani*, and *Asaphestra intermedia*⁷. Data on *Eocaecilia micropodia* and *Proterogyrinus scheelei* are taken from composite skeletal materials.

separate quadratojugal present; maxilla separate from palatine; large orbit; intercentra and limbs present.

The skull of *Eocaecilia micropodia* shares certain distinctive features with modern caecilians thought to be adaptations to fossoriality: a compact skull roof, well ossified braincase and robust jaws. The braincase is co-ossified as the os basale, as in all living caecilians. A definitively caecilian character is a depression along the anteroventral margin of the orbit that we interpret as a tentacular fossa (T, Fig. 2b). The tentacle of living species is generally believed to serve chemosensory and possibly tactile functions and incorporates both vomeronasal and Harderian glands as well as orbital structures⁴; the aperture of the tentacle varies in position from beneath the external naris to within the orbit. Rather than a deep sulcus or an entirely separate bony canal, the tentacular fossa of *E. micropodia* is only a shallow incisure along the orbital margin (Fig. 1a), similar to that in rhinatrematids⁵. In contrast to the modern condition, the orbits of *E. micropodia* have not undergone reduction in size.

The dermal palate is typically caecilian (Figs 1b and 2c). The presence of separate palatines, unfused to the maxillae, is unique to *Eocaecilia micropodia* among known gymnophiones. There is no ectopterygoid. The parasphenoid, which bears numerous teeth, is fully fused to the rest of the os basale. A few palatal pedicels are also found on the posteromedial region of the pterygoid. The interpterygoid vacuities are longer and wider than is normally the case in modern caecilians. There are two arcades of pedicellate teeth, the outer borne on the maxilla and premaxilla (40 teeth estimated), the inner on the palatine and vomer (30 teeth estimated). The large internal choana is medial to the inner dental arcade. No specimen preserves tooth crowns in place on the pedicels. But tooth crowns found in direct association with cranial material are conical, recurved and bicuspedate (Fig. 1c, d); the asymmetry of the crests leading from the secondary cusp and the general form of the teeth resemble those in some modern taxa (for example *Ichthyophis glutinosus*, *Uraeotyphlus narayani*, *Hypogeophis rostratus*, *Geotrypetes seraphini*⁶).

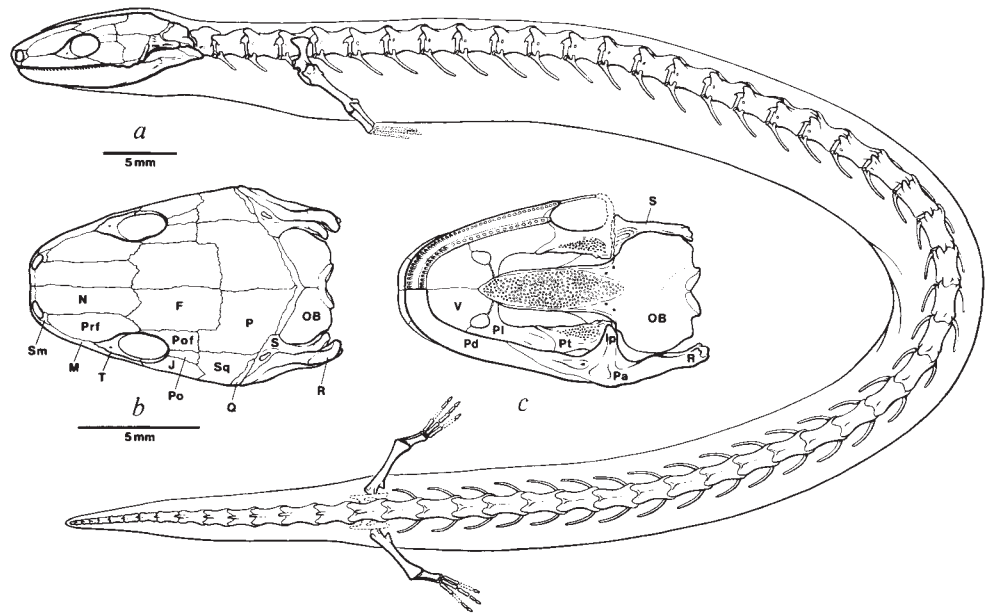
The stapes in modern caecilians typically consists of a large, ovoid footplate and a short, anteriorly directed stapedial process that articulates with the quadrate. In contrast, the stapes of *Eocaecilia micropodia* is uniquely configured. The anterior end, pierced by a stapedial foramen, is expanded both mediolaterally and dorsoventrally, and extensively contacts both the quadrate and braincase (S, Fig. 2b, c). The posterior end, representing the footplate apposed to a large fenestra ovalis, also articulates with the retroarticular process of the lower jaw (Fig. 1a). An oval, slightly rugose facet on the medial surface of the retroarticular process and a similar facet on the corresponding lateral surface of the footplate appear to represent a synovial joint between the two bones (Fig. 1e).

The lower jaw of *Eocaecilia micropodia* is slightly subterminal. As in other caecilians, the jaw comprises two bones, the pseudo-dentary and the pseudoangular (Pd, Pa, Fig. 2c), joined along a squamous suture that crosses obliquely from anteromedial to posterolateral sides. The pseudoangular exhibits large retroarticular and internal processes (R, IP, Fig. 2). The pseudodentary bears two rows of small pedicellate teeth, about 40 on the outer row, 20 on the inner.

The atlas (Fig. 1f) bears two large cotyles for articulation with the occipital condyles. Between the cotyles is a well developed interglenoid tubercle, a feature not present in any living caecilian but which occurs in microsaur⁷ and salamanders. The posterior half of the centrum is deeply excavated by a notochordal fossa. In place of a spinous process is an elongate, low tuberosity that bifurcates posteriorly.

Postatlantal vertebrae of Recent caecilians, which range in number from about 80 to 285, typically possess a ventral, longitudinal keel along an hourglass-shaped, amphicoelous body, a distinct, anteriorly placed diapophysis and parapophysis for articulation with the costal tubercle and head, and an anteriorly projecting parapophysal process⁸. Spinous processes are absent. On the anterior 10 to 20 vertebrae, a nuchal keel is developed for the attachment of dorsal trunk muscles, and an intravertebral foramen for a spinal nerve passes through the anterior part of

FIG. 2 a, Reconstruction of *Eocaecilia micropodia*, gen. et sp. nov. The anterior half of the body is shown in lateral view, the posterior half in dorsal view. Details of the skull are shown in dorsal (b) and ventral (c) views. In c the left jaw and right stapes have been removed. F, Frontal; Ip, internal process; J, jugal; M, maxilla; N, nasal; OB, os basale; P, parietal; Pa, pseudoangular; Pd, pseudodentary; Pl, palatine; Pm, premaxilla; Po, postorbital; Pof, postfrontal; Prf, prefrontal; Pt, pterygoid; Q, quadrate; R, retroarticular process; Sm, septomaxilla; Sq, squamosal; S, stapes; T, tentacular fossa (on the lacrimal bone); V, vomer. By analogy with that in modern forms, the large internal process (Ip, in c) would have served as a trochlea redirecting the course of the levator posterior part of the mandibular adductor muscle from the subtemporal fenestra to the ventromedial surface of the retroarticular process¹⁷. The retroarticular process, half the length of the pre-articular portion of the jaw, is sufficiently similar to those of modern forms that we infer that the interhyoideus posterior muscle already functioned as part of the uniquely caecilian mechanism of jaw closure in which the muscle's posteroventrally directed traction on the process produces the effect of a third-order lever^{17,18}. The robustness of the stapes and its intercalated position between the retroarticular process and braincase appear to represent a buttressing mechanism against the medially directed, compressive force generated by the interhyoideus posterior during biting. Although the details of the postcranial axial skeleton of *E. micropodia* are clearly that of a primitive caecilian,



the extent of truncal elongation remains uncertain because no specimen preserves a complete, articulated column. On the basis of the most complete specimens, the minimum number of presacral vertebrae is estimated to be 42. A series of three vertebrae, possibly sacral, are associated with broad, single-headed ribs; relatively robust diapophyses are situated on the pedicles where intravertebral foramina normally occur. Caudal vertebrae, in contrast to dorsal vertebrae, have spinous processes.

the pedicle. Short, bicapitate ribs occur on all but the most posterior vertebrae. *Eocaecilia micropodia* possesses several of these features, notably amphicoely, intravertebral foramina, short, bicapitate ribs and lack of spinous processes (Figs 1g and 2a). But ventral keels are only incipient, and elaborate diapophyseal and parapophyseal processes are not developed. The lengths of dorsal vertebral centra vary from 1.5 to 2.2 mm; the longest vertebrae are thus 17% of estimated average skull length, which is comparable to the proportions of modern species (Table 1). A feature of *E. micropodia* not found in any living amphibian (but present in many Palaeozoic forms) is intercentra (arrows, Fig. 1g).

Scapulocoracoids (Figs 1g and 3a), humeri (Figs 1g and 3b), radii, ulnae (Fig. 1g), femora (Fig. 1h), tibiae and fibulae have been identified. Of the three specimens that preserve partially disarticulated feet (one is identifiable as a pes by its association with a hindlimb), none shows more than three digits. Pelvic elements have yet to be found. The ultimate loss of limbs in later caecilians raises the question of whether those of *Eocaecilia micropodia* were undergoing reduction. Limb elements of *E. micropodia* are proportionately shorter than those of terrestrial Palaeozoic amphibians such as *Proterogyrinus scheelei*, and various microsaur (which are comparable to *E. micropodia* in body size; Table 1). Compared to representatives of living salamander families, *E. micropodia* is closest in limb proportions to shorter-limbed forms (*Necturus maculosus*, *Cryptobranchus alleganiensis*, *Desmognathus* spp., *Pseudotriton montanus*; Table 1). Thus, some reduction of the appendicular skeleton appears to have taken place.

The evolutionary origin of Recent amphibians—frogs, salamanders and caecilians—has been a persistent problem because the fossil record of their emergence is unknown and because each group is structurally so highly derived in comparison to

Palaeozoic forms. Prevailing opinion is that the three Recent orders constitute a monophyletic group, the Lissamphibia, but several alternative phylogenetic hypotheses have been proposed^{9,10}. The discovery of a well preserved series of fossil caecilians now provides potentially decisive material for resolving this issue. *Eocaecilia micropodia* already adds substantial information on the history of modern amphibians. First, the Early Jurassic age of *E. micropodia*, which is older than the earliest known salamanders (Middle Jurassic)¹¹ but younger than the earliest known frogs (Late Triassic)¹², confirms that the three groups were well differentiated by mid-Mesozoic times and thus all probably originated during the Triassic or even earlier. Second, the origin of the vertebral structure in modern amphibians has been problematic because heretofore the homology of the holospondylous centrum could not be determined¹³. The presence of conventional, small intercentra in *E. micropodia* is evidence that the primary central element of gymnophiones, at least, is a pleurocentrum. Third, the structure of the tooth crown in modern caecilians varies from monocuspate to bicuspate, and from conical to spatulate⁶. Bicuspate teeth have been presumed to be primitive because this pattern is widespread among frogs, salamanders and caecilians¹⁴; the bicuspate teeth of the Jurassic caecilian provide additional support to this hypothesis. Fourth, *E. micropodia* reveals features that have been lost in Recent caecilians. The structure of the proximal femur and the presence of an interglenoid tubercle strengthens the putative relationship of caecilians and salamanders. Although the femoral features clearly differ from those known in Palaeozoic amphibians, an interglenoid tubercle is characteristic of microsaur alone among Palaeozoic groups. Last, among living caecilians, the most primitive group recognized by cladistic analysis is the Rhinatrematidae. The two genera included in this family have an open (zygokrotaphic) skull roof in the temporal region, a condition

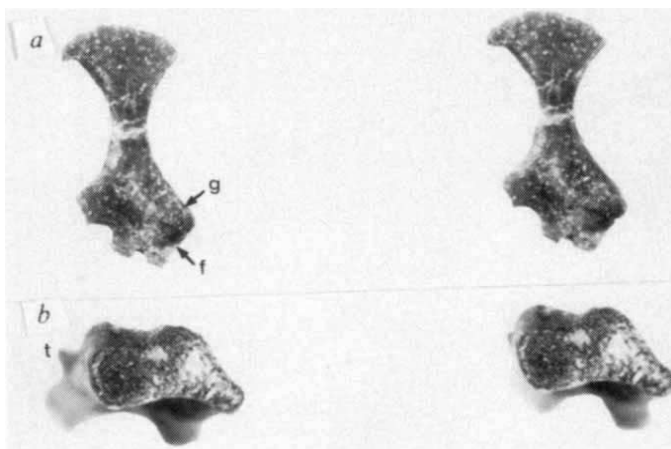


FIG. 3 Stereoscopic photographs, *Eocaecilia micropodia*, gen. et sp. nov. a, Lateral view of a left scapula (MCZ 9238A, $\times 13.2$). The rough texture of the superior margin is evidence of a suprascapular cartilage. The anterior (scapular) part of the elongate glenoid (g), which faces posteroventrolaterad, is preserved; the posterior (coracoid) part, which faces primarily dorsad and slightly laterad, is missing from this specimen (see Sc. Fig. 1g). Anteroventral to the glenoid is a circular fossa (f), as in some microsaur⁷. The coracoid foramen, represented by a deep notch in the anterior margin of the coracoid, was probably completed by cartilage. b, Proximal view of a humeral head (MCZ 9171A, $\times 18.5$). The bulbous articular facet, which extends from the dorsolateral to the ventromedial surfaces, exhibits a spiral shape similar to that in Palaeozoic amphibians. A ventrally reflected deltopectoral crest is continuous with a ridge that extends proximally. On the medial side of the diaphysis is a small tuberosity (t) comparable in position to that in some microsaur⁷ as well as in Recent salamanders (where it serves as the insertion of subscapular musculature).

hypothesized to be primitive^{5,15} and shared with salamanders and frogs. The occurrence of a solidly roofed (stegokrotaphic) skull in an Early Jurassic caecilian, which is otherwise primitive in a number of other cranial characters, strengthens the possibility that the zygokrotaphic condition may be derived. □

Received 7 April; accepted 7 July 1993.

1. Duellman, W. E. & Trueb, L. *Biology of Amphibians* (McGraw-Hill, New York, 1986).
2. Estes, R. & Wake, M. H. *Nature* **239**, 228–231 (1972).
3. Rage, J.-C. C. r. *Acad. Sci. Paris Ser. II* **302**, 1033–1036 (1986).
4. Billo, R. & Wake, M. H. *J. Morph.* **192**, 101–111 (1987).
5. Nussbaum, R. A. *Occ. Pap. Mus. Zool. Univ. Mich.* **682**, 1–30 (1977).
6. Wake, M. H. & Wurst, G. Z. *J. Morph.* **159**, 331–341 (1979).
7. Carroll, R. L. & Gaskill, P. *Mem. Am. phil. Soc.* **126**, 1–211 (1978).
8. Wake, M. H. *J. Morph.* **165**, 117–130 (1980).
9. Carroll, R. L. & Currie, P. J. *Zool. J. Linn. Soc.* **57**, 229–247 (1975).

10. Trueb, L. & Cloutier, R. in *Origins of the Higher Groups of Tetrapods: Controversy and Consensus* (eds Schultze, H.-P. & Trueb, L.) 223–313 (Comstock, Ithaca, 1991).
11. Evans, S. E., Milner, A. R. & Mussett, F. *Geobios* **21**, 539–552 (1988).
12. Rage, J.-C. & Roček, Z. *Palaeontographica (Abt. A)* **206**, 1–16 (1989).
13. Wake, D. B. & Lawson, R. J. *Morph.* **139**, 251–298 (1973).
14. Wilkinson, M. Z. *zool. Syst. Evolut.-forsch.* **29**, 304–311 (1991).
15. Wake, M. H. & Hanken, J. J. *Morph.* **173**, 203–223 (1982).
16. Holmes, R. *Phil. Trans. R. Soc.* **B306**, 431–524 (1984).
17. Bemis, W. E., Schwenk, K. & Wake, M. H. *Zool. J. Linn. Soc.* **77**, 75–96 (1983).
18. Nussbaum, R. A. *J. Zool. Lond.* **199**, 545–554 (1983).

ACKNOWLEDGEMENTS. We thank W. W. Amaral for specimen preparation, A. H. Coleman for stereophotography, R. Pinto for scanning electron microscopy, J. P. O. Rosado for herpetological materials in the Museum of Comparative Zoology, K. A. Joysey and J. A. Clack for access to the Museum of Zoology, University of Cambridge, R. C. Carroll, A. C. Milner, R. A. Nussbaum, J. O. Reiss and M. H. Wake for advice and W. W. Amaral, W. R. Downs, S. Madsen, R. J. O'Hara, T. B. Rowe, C. R. Schaff and N. H. Shubin for field collection of the Kayenta caecilians. Supported by grants from the National Geographic Society and NSF (F.A.J.) and the Bedford Fund for Zoological Research, King's College, Cambridge (D.M.W.).

The time course of learning a visual skill

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SEVERAL examples of experience-dependent perceptual improvement (perceptual learning) suggest that plasticity in specific neuronal loci could underlie the learning process^{1–6}. For a basic visual discrimination task (using an optimal stimulus for 'automatic' pre-attentive texture segregation^{7,10}), discrete retinal input-dependent changes within a very early stage in the stream of visual processing were indicated as the locus of a large and consistent learning effect⁵. When do these changes occur? Here we report that except for a fast, rapidly saturating improvement early in the first practice session, performance was very stable within sessions. Indeed, observers showed little or no improvement until up to 8 hours after their last training session (latent phase). But large improvements occurred thereafter. Finally, there was almost no forgetting; what was gained was retained for at least 2–3 years. We conjecture that some types of perceptual experience trigger permanent neural changes in early processing stages of the adult visual system. These may take many hours to become functional.

Nine observers took part in these experiments. Stimulus parameters and procedure were as previously described⁵ (Fig.

1). The observers' task was to decide whether a small target texture—an array of 3 diagonal line elements (bars) differing only in their orientation from a background of identical elements—was horizontal or vertical (Fig. 1a). Performance was measured as the mean per cent correct response for increasingly shorter time intervals between the briefly presented (10 ms) stimulus and a patterned mask (stimulus-to-mask onset asynchrony, SOA). A psychometric curve was then constructed, from which a threshold SOA for 80% correct discrimination could be derived (Fig. 1b, c). As previously reported⁵, the main effect of practice is a leftward shift of the performance curves, indicating genuine increases in sensitivity, on consecutive sessions spaced 1–3 days apart. Where perception completely fails on the initial session, there is >90% correct discrimination on the following day (Fig. 1b). Thus the increments in performance refer to learning that was retained from one daily session to the next.

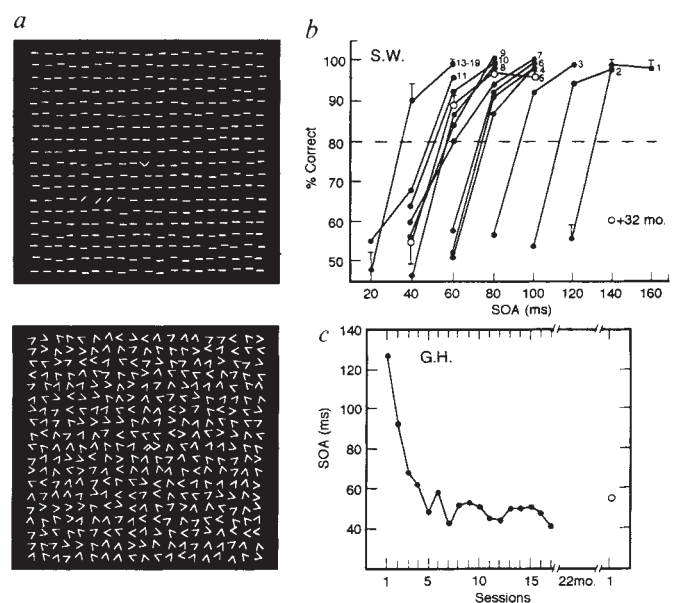
For most observers, a fast learning effect which quickly saturates could be shown (Fig. 2a). But this consistently occurred only during the first few practice blocks of the very first session or two in naive observers, and only with above-threshold (as defined by the psychometric curve for the session) novel stimuli. Following this short phase, performance was very stable for consecutive blocks of trials, and no amount of practice affected the psychometric curves or improved the discrimination threshold within a session (Fig. 2). Yet the psychometric curves (and perceptual thresholds) continued to improve, as shown on subsequent daily sessions (Figs 1b, c and 2f, g). Also, unlike the between-sessions improvement in discrimination thresholds⁵, 'fast' learning did transfer from a trained eye to the other (although it too was specific for orientation and visual-field location).

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FIG. 1 Computer-generated displays. **a** Top, a test stimulus with a small target texture (3 diagonal bars) embedded within a background of horizontal elements. Display parameters were set as previously described⁵. Display size was 14° by 14° of visual angle viewed from a distance of 110 cm, with an array of 19 by 19 slightly jittered line segments (subtending $0.42^\circ \times 0.03^\circ$ each, with a luminance of 35 cd m^{-2} and spaced 0.70° apart). A small rotated letter (either T or L) at centre of display served as the fixation target. The target texture's position was varied randomly from trial to trial but always within a specific display quadrant and within 2.5° – 5° of visual angle from centre of display. Bottom, mask pattern made of randomly oriented V-shaped micropatterns and at the centre, a compound micropattern of superimposed T and L. **b**, Psychometric curves for performance on consecutive daily sessions (spaced 1–3 days apart) for observer S.W. (●), and on a probe session 32 months later (○) (S.W. had no further psychophysical experience with visual textures in the interval, although she participated in some experiments involving random-dot stereogram depth perception). Results indicate persistent day-to-day improvements and almost no forgetting on a timescale of years. Each data point represents the mean per cent correct responses ($\pm$ s.d.) from 3–5 consecutive blocks (150–250 trials) for a specific SOA. The initial performance curve is on the right; as learning occurs, the curves are displaced to the left (shorter processing times needed for task performance), indicating improved sensitivity. The far left curve represents asymptotic performance. Dashed line, 80% correct (threshold) performance. **c**, Learning curve for observer G.H. SOA required for threshold discrimination on consecutive daily sessions (●), and for a probe session 22 months later (○). Each point refers to a single session, interpolated from the respective psychometric curve. The large learning effect has not decayed in the interval (where no psychophysical testing or training was done).

METHODS. Each session consisted of 16–24 blocks of 50 trials (stimulus presentations) each. Observers were instructed to fixate a small central cross and then activate the trial sequence: blank screen interval (250–300 ms), the stimulus (10 ms), blank inter-stimulus interval (calculated from the onset of the stimulus display, SOA), the mask (100 ms), blank screen until response (no time limit). For each display the observers were required first to identify the letter at fixation point and then to decide whether the foreground texture target was vertical or horizontal. Immediate auditory feedback was given only for the fixation



control task. Because stimuli were presented for only 10 ms, no eye movement could displace the stimulus on the retina, ensuring that the target texture consistently appeared in a specific retinotopic location. In the initial session for each observer, the SOA was set at 240–300 ms to establish above 95% correct texture discrimination. On all following sessions the initial SOA was set to the lowest SOA, for which 95% correct discrimination was obtained in the previous session. Then, on each session, 3–5 consecutive blocks of trials were run per SOA, which was then decreased by a step of 20 ms and another group of 3–5 blocks were run. This successive stepwise reduction in SOA was repeated until perception failed (less than 60% correct discrimination on 3–4 consecutive blocks).

If perceptual performance does not change during the training session, when does learning occur? To follow the time course of this change, the test procedure was repeated in probe sessions spaced from 20 minutes to 10 hours after the completion of the initial training session of the day. In Fig. 3, the gain in performance for 9 observers is plotted as a function of time elapsed since the termination of the practice session. No improvement was found on retesting within the first 8 hours (latent phase). Only after 8 hours did some observers show a learning effect. By

the following day, however, large improvements had occurred (discrimination threshold lowered by 43 ± 11 ms (mean, s.d.). The additional practice during the latent phase did not induce larger gains than those found in our original observations with no intermediate probe sessions (38 ± 7 ms (mean, s.d.), 5 observers) (*t*-test, n.s.). This suggests that training during the latent phase was superfluous, and that learning was driven by the sensory experience acquired at the initial session.

Once discrimination thresholds have improved, there seems to

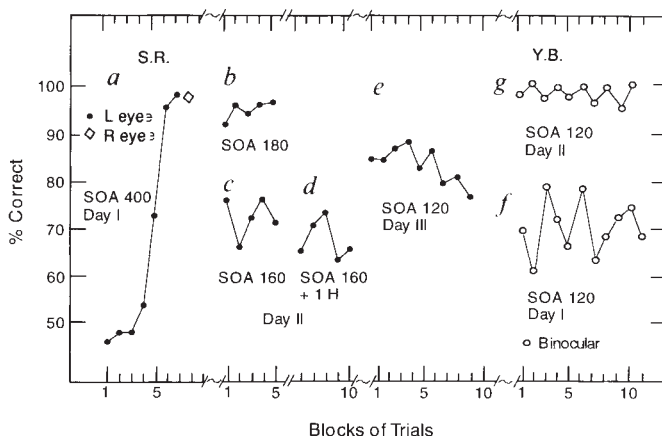
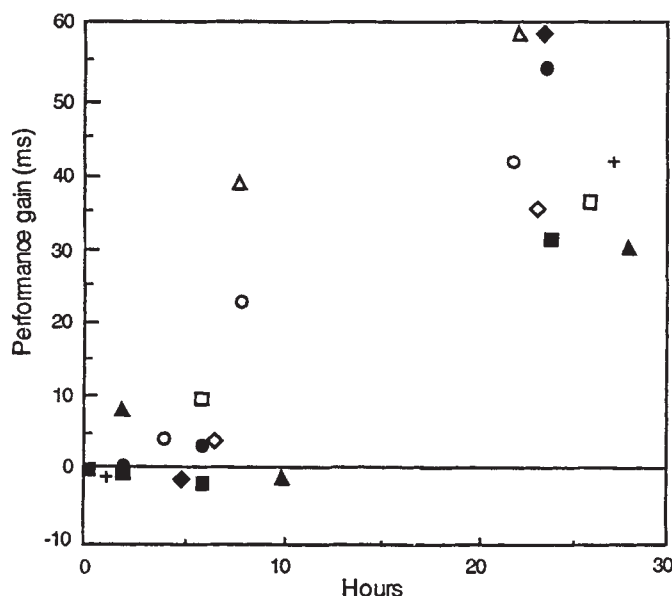


FIG. 2 Fast (within-session) and slow (between-session) learning. Performance for consecutive blocks of 50 trials each, on consecutive daily sessions. The procedure was modified in that the initial SOA was set to 260–400 ms (two to three times the previously determined mean threshold for 10 naive observers) and was not changed until performance stabilized. The completion of a single block of trials took 2–4 min, so performance changes within a timescale of a few minutes were assayed. **a–d**, Observer S.R. **a**, Fast improvement on the very first blocks of trials at above threshold stimulus presentation (SOA at 400 ms). ●, Left-eye viewing; ◇, right-eye viewing. Unlike the slow learning effect⁵, there is complete interocular transfer of the fast learning effect. **b, c**, Stable performance above and below threshold (SOA at 180 and 160 ms, respectively) indicating saturation of the fast learning effect by the second session. **d**, An hour later performance is stable and there is no improvement (SOA at 160 ms). **e**, 20 h later, performance at 120 ms SOA shows a more than 40 ms gain to have occurred in the interval from (**d**). **f, g**, Observer Y.B. SOA is at 120 ms. Performance is very stable within each session, but there is marked improvement on retesting after an interval of 24 h. **f**, Performance at threshold on the first training session, following above threshold experience and the saturation of the fast learning effect. **g**, Performance 24 h after the initial session.

FIG. 3 Slow (latent) improvement in perception several hours after visual experience was terminated. Performance gain (in terms of threshold SOA, in ms, each extrapolated from a complete psychometric curve (see Fig. 1) at different time intervals from the initial training session (normalized at zero). Nine observers. Each symbol represents a single observer exposed to a set stimulus configuration, probed 1–3 times in the first 10 h interval after the initial session, and once again after an interval of 20–30 h. Two observers ($\circ$, Δ) show a performance gain of more than 20 ms, after an 8 h interval whereas another ($\blacktriangle$) is still within the latent phase after 10 h. For up to 6 h there is no gain in performance (mean change, 2 ± 5 ms). By 22–28 h after the initial training session all observers have gained in visual sensitivity. The gain was not related to the number of intervening test-practice sessions. The gap in data for the time interval between 10 and 20 h after the training session, stands for night time. Experiments to be reported in detail elsewhere have shown that the process of consolidation occurs also during normal sleep, and depends on the integrity of stage REM sleep²².



be no forgetting over periods of many months. Figure 1b depicts the discrimination performance of S.W. on consecutive daily sessions during September–October 1989, and her performance on a probe session 32 months later, with no training in the interval. Almost three years later most of the gain was retained. This was also the case for G.H., after an interval of 22 months (Fig. 1c).

It has been assumed that perceptual improvement closely follows practice, and the ability to detect or discriminate visual stimuli improves over the course of the training session; some tasks require more training^{2,4}, whereas for others the effects of practice occur rapidly, within a few tens of trials^{1,3,6}. Long-term perceptual learning effects, on a slower timescale, have also been ascribed to within-session improvements carried over from one session to the next^{2,4,8,11}. But perhaps for the first time, our results show that not all human learning is concurrent with practice. The psychophysical evidence suggests that two stages, possibly two processes, subserve texture discrimination learning. Each has a distinct time course (evolution and saturation) and although both show a high degree of stimulus specificity, only the slow, between-sessions, learning effect is monocular. This finding implies that slow learning is mediated by earlier stages of processing^{12,13}. Although fast learning may reflect the setting up of a task-specific routine^{6,14} for solving the perceptual problem, slow learning may indicate the ongoing long-term, perhaps structural, modification of basic perceptual modules.

The main result uncovered by following the time course of texture discrimination learning is a latent phase of several hours duration, during which the perceptual skill evolves. This result has now been observed in two other examples of perceptual

learning. For untrained observers, contrast detection is improved when masks are positioned at a short distance from the target¹⁵. But, this distance can be more than tripled by practice, a learning effect that shows strong retinotopy, orientation-specificity and monocularity¹⁶ and follows a two-phased time course with a latent phase of about 8 hours duration (U. Polat and D.S., manuscript in preparation). Second, although fast stereo-fusion learning has been previously described¹, a slow, between-session improvement in stereo-fusing random dot displays across increasing disparities has been recently found (I. Kovács and B. Julesz, personal communication). In all three examples, long-term, experience-driven improvements in the perceptual skill occur following a latent period of several hours. We suggest the term 'consolidation' for the process, presumably initiated during the practice session, which underlies the improvement of perceptual sensitivity several hours after visual experience was terminated. Consistent with this interpretation of our human data are the kinetics of early-life (critical period) cortical plasticity¹⁷, and patterns of mammalian memory consolidation that have shown a latent phase of several hours before the long-term retention of a specific skill^{18,19}. One example of a possible neural mechanism of latent-phase learning is given by the experience-induced (imprinting) changes in NMDA receptor binding in chick brain which follows a latent phase of 6–8 hours²⁰. This may be pertinent to perceptual learning, because NMDA receptor blockade had been shown to disrupt mammalian visual cortex plasticity²¹. We conjecture that slow learning reflects a functional property of basic neuronal mechanisms of memory storage within the adult sensory system. These may subserve the long-term retention of some perceptual skills. □

Received 30 April; accepted 24 June 1993.

- Ramachandran, V. S. & Braddick, O. *Perception* **2**, 371–376 (1973).
- McKee, S. P. & Westheimer, G. *Percept. Psychophys.* **24**, 258–262 (1978).
- Fiorini, A. & Berardi, N. *Vision Res.* **21**, 1149–1158 (1981).
- Bail, K. & Sekuler, R. *Vision Res.* **27**, 935–965 (1987).
- Karni, A. & Sagi, D. *Proc. natn. Acad. Sci. U.S.A.* **88**, 4966–4970 (1991).
- Poggio, T., Fahle, M. & Edelman, S. *Science* **256**, 1018–1021 (1992).
- Julesz, B. in *Dynamic Aspects of Neocortical Function* (eds Edelman, G. M., Cowan, W. M. & Gall, W. E.) 585–612 (Wiley, New York, 1984).
- Beck, J. (ed.) in *Organization and Representation in Perception* 285–317 (Erlbaum, Hillsdale, New Jersey, 1982).
- Nothdurft, H. C. *Vision Res.* **25**, 551–560 (1985).
- Sagi, D. & Julesz, B. *Science* **228**, 1217–1219 (1985).
- Gurnsey, R. & Browse, R. A. *Percept. Psychophys.* **41**, 239–252 (1987).
- Zeki, S. M. *Nature* **274**, 423–428 (1978).
- Hubel, D. *Nature* **299**, 515–524 (1982).
- Ullman, S. *Cognition* **18**, 97–157 (1984).
- Polat, U. & Sagi, D. *Vision Res.* **33**, 993–999 (1993).
- Polat, U. & Sagi, D. *Invest. Ophthalmol. Vis. Sci.* **34** (suppl.), 776 (1993).
- Buisseret, P., Gary-Bobo, E. & Imbert M. *Nature* **272**, 816–817 (1978).
- McGaugh, J. L. *Science* **153**, 1351–1358 (1966).
- Matthies, H. *Progr. Neurobiol.* **32**, 277–349 (1989).
- Horn, G. & McCabe, B. J. *J. Physiol.* **423**, 92 (1990).
- Bear, M. F., Gu, Q., Kleinschmidt, A. & Singer, W. *J. Neurosci.* **10**, 909–925 (1990).
- Karni, A. & Sagi, D. *Neurosci. Abstr.* **18**, 387 (1992).

ACKNOWLEDGEMENTS. We thank M. Mishkin, L. Ungerleider, R. Desimone, B. Richmond, L. Chelazzi and P. De Weerd for discussions and suggestions, and Y. Barbut and M. Adams for graphical help. This work was supported by the Israeli Center for Psychobiology.

Retrograde axonal transport of ciliary neurotrophic factor is increased by peripheral nerve injury

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CILIARY neurotrophic factor (CNTF) promotes the survival of several populations of neurons, including sensory and motor neurons¹⁻⁴. Although CNTF is abundant in adult sciatic nerve, the mature protein lacks a signal sequence and is not secreted; therefore, it has been proposed to act as a lesion factor³. The identification of a functional CNTF receptor revealed ligand-specific phosphorylation cascades and gene induction⁵. However, it is not clear how these signal-transducing events are elicited in neuronal cell bodies that may be distant from the source of CNTF. We report here that CNTF can be retrogradely transported by adult sensory neurons. More importantly, sensory and motor neurons both show greatly increased transport of CNTF following peripheral nerve lesion. Axotomy-induced increases in retrograde transport of neurotrophic factors may be an important response of neuronal cell bodies during regeneration.

CNTF is expressed at very high levels in peripheral nerve whereas little or none can be detected in muscle or other targets of sensory and motor neurons^{3,6,7}. We therefore chose to study the retrograde transport of CNTF after intraneural injection to approximate the physiological conditions under which the axons of sensory and motor neurons are exposed to CNTF. After injection into intact sciatic nerve of adult rats, ¹²⁵I-labelled CNTF was retrogradely transported to sensory neurons in 4th and 5th lumbar (L4, L5) dorsal root ganglia (DRG) (Fig. 1). When the sciatic nerve was crushed 7 days before ¹²⁵I-CNTF injection, retrograde transport to sensory neurons was increased fivefold. Transport was specific, as shown by the absence of labelled CNTF in the DRG contralateral to the injection site and by the ability of excess unlabelled CNTF to block accumulation. The CNTF receptor complex consists of a CNTF binding protein (CNTFR- α)⁸, and two other proteins, LIFR- β and gp130. By themselves, LIFR- β and gp130 constitute a functional leukaemia inhibitory factor (LIF) receptor that can be converted to a functional CNTF receptor by CNTFR- α ^{5,7,10}. As predicted from these observations, ¹²⁵I-CNTF transport in lesioned sciatic nerve was also inhibited by LIF. Conversely, interleukin-6 (IL-6), a related cytokine that does not bind directly to the CNTF receptor complex, but uses gp130 as a signal transducing component¹¹, did not block transport (Fig. 1). A similar pharmacologic profile was obtained with unlesioned animals (not shown). No transport was observed after heat inactivation of ¹²⁵I-CNTF or nerve ligation proximal to the injection site (not shown). Collectively, these results show that CNTF undergoes receptor-mediated retrograde axonal transport to DRG neurons.

Emulsion autoradiography was used to identify populations of sensory neurons retrogradely transporting ¹²⁵I-CNTF. In unlesioned animals, silver grains were apparent over the cell bodies of some small DRG neurons, with minimal labelling of large cells (Fig. 2a). Labelling was abolished by coinjection of excess unlabelled CNTF or LIF, but not IL-6 (not shown), consistent with the quantitative results (Fig. 1). Prior nerve crush increased the labelling of both small and large neurons, such that labelled large neurons were more readily apparent (arrow

in Fig. 2b). Size-frequency analysis (Fig. 2c, d) demonstrated significantly increased frequency of labelling of larger neurons (900–2,000 μm^2) after lesion. Although there was a decrease in the fraction of labelled cells less than 900 μm^2 , the absolute number of labelled small cells in lesioned animals was increased.

After injection of ¹²⁵I-CNTF into sciatic nerve, low levels of radioactivity were routinely detected in the spinal cord of unlesioned animals (Fig. 1) but this could not be diminished by coinjection of excess unlabelled CNTF (not shown). This suggests that ¹²⁵I-CNTF had accumulated nonspecifically, as supported by the absence of silver grains over motor neurons in unlesioned animals (Fig. 2e). However, prior nerve crush resulted in retrograde transport of ¹²⁵I-CNTF to the spinal cord (Fig. 1) and this was corroborated by the appearance of numerous silver grains over most (66% $\pm$ 4%) motor neuron cell bodies (Fig. 2f). Retrograde transport to lesioned motor neurons was receptor mediated, as coinjection of excess unlabelled CNTF or LIF, but not IL-6, blocked transport (not shown).

There are many possible reasons to explain increased CNTF retrograde transport caused by nerve lesion, as well as why motor neurons and large sensory neurons only become labelled after axonal injury. First, there may be induction of retrograde transport in previously non-responsive cells. This could be achieved by increased expression of CNTF receptor subunits or redistribution of existing receptors by injured neurons. Although CNTF retrograde transport is elevated as early as 1 day after lesion, northern blot analysis showed little, if any, change in expression of the CNTF receptor components, CNTFR- α , gp130 and LIFR- β in DRG up to 14 days after sciatic nerve crush (Fig. 3). Second, CNTF may have greater access to axonal elements following a lesion. This could be affected by loss of

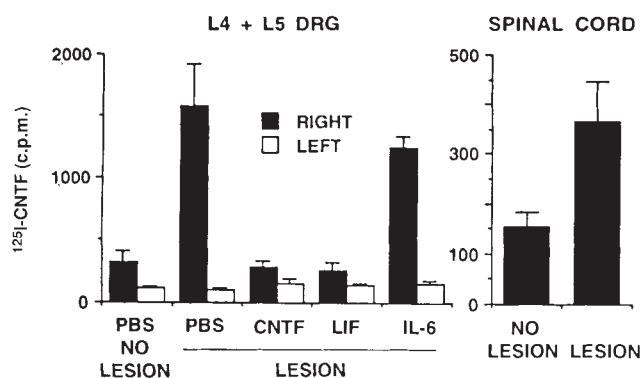


FIG. 1 Retrograde transport of ¹²⁵I-CNTF from sciatic nerve to lumbar DRG is increased by prior nerve lesion and can be blocked by excess unlabelled CNTF or LIF (but not IL-6). ¹²⁵I-CNTF accumulation in spinal cord is also increased by prior nerve lesion.

METHODS. Recombinant rat CNTF²⁵ was iodinated to specific activities of 2,000–2,500 counts per minute (c.p.m.) fmol⁻¹ (ref. 26). ¹²⁵I-CNTF retained 70–80% of its biological activity as assessed in an embryonic chick ciliary neuron survival assay²⁵. In all animals, injections containing 1 μl ¹²⁵I-CNTF (44 ng; 3.5 μCi) and 1 μl of PBS or other factors (CNTF, LIF, IL-6 dissolved in PBS) were made into the right sciatic nerve at the level of the tendon of the obturator internus muscle in adult male Sprague-Dawley rats (150–200 g) anaesthetized with 170 mg kg⁻¹ chloral hydrate and 35 mg kg⁻¹ pentobarbital. For lesion studies, the sciatic nerve was crushed at the obturator tendon and injections were made directly into the crush site 7 days later. For competition experiments, excess unlabelled CNTF or LIF (20-fold) or IL-6 (45-fold) was coinjected with ¹²⁵I-CNTF. DRG were dissected 6 h after the ¹²⁵I-CNTF injection, and spinal cord was removed at 12 h. All tissues were immersion-fixed in 4% paraformaldehyde/PBS and radioactivity was quantified in a gamma counter. Counts per min from spinal cord, and right (solid bars) or left (open bars) L4 + L5 DRG were expressed as mean $\pm$ s.d., $n=4-5$; results are representative of 3–5 such experiments.

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FIG. 2 Autoradiographic visualization of ^{125}I -CNTF after retrograde transport to sensory and motor neurons in normal animals and 7 days after sciatic nerve crush. Bright field photomicrographs of *a*, normal and *b*, lesioned sensory neurons after retrograde accumulation of ^{125}I -CNTF. Note the greater intensity of cellular labelling, as well as marked labelling of very large DRG neurons (arrow in *b*) after lesion. Corresponding size-frequency histograms of labelled DRG neurons from *c*, normal and *d*, lesioned rats. A cell was considered labelled if both a defined nucleolus was visible and the grain density over the cytoplasm was at least twice background. Per cent labelling at size ranges between $900\text{--}1,260\ \mu\text{m}^2$ and $1,380\text{--}2,040\ \mu\text{m}^2$ was significantly increased with lesion ($P < 0.035$; ANOVA, Scheffe's test). Dark-field photomicrographs showing retrogradely transported ^{125}I -CNTF in spinal cord motor neurons of *e*, normal and *f*, lesioned rats. Labelled motor neurons were never apparent in normal rats (*e*). Scale bar in *a*, $25\ \mu\text{m}$; in *e*, $50\ \mu\text{m}$.

METHODS. Fixed tissue was equilibrated in 30% sucrose and frozen sections were cut at $10\ \mu\text{m}$. Slides were dipped in Kodak NTB2 emulsion, exposed for 13 weeks at 4°C and developed in D19 (Kodak) and counterstained with 0.5% cresyl violet. Areas of labelled cells were determined using a JAVA planimetry program (Jandel Scientific). Areas were grouped into $60\text{-}\mu\text{m}^2$ intervals and the per cent of total labelled cells for each interval was expressed as mean $\pm$ s.e., $n = 3$ (total cells counted: *c*, 651; *d*, 779).

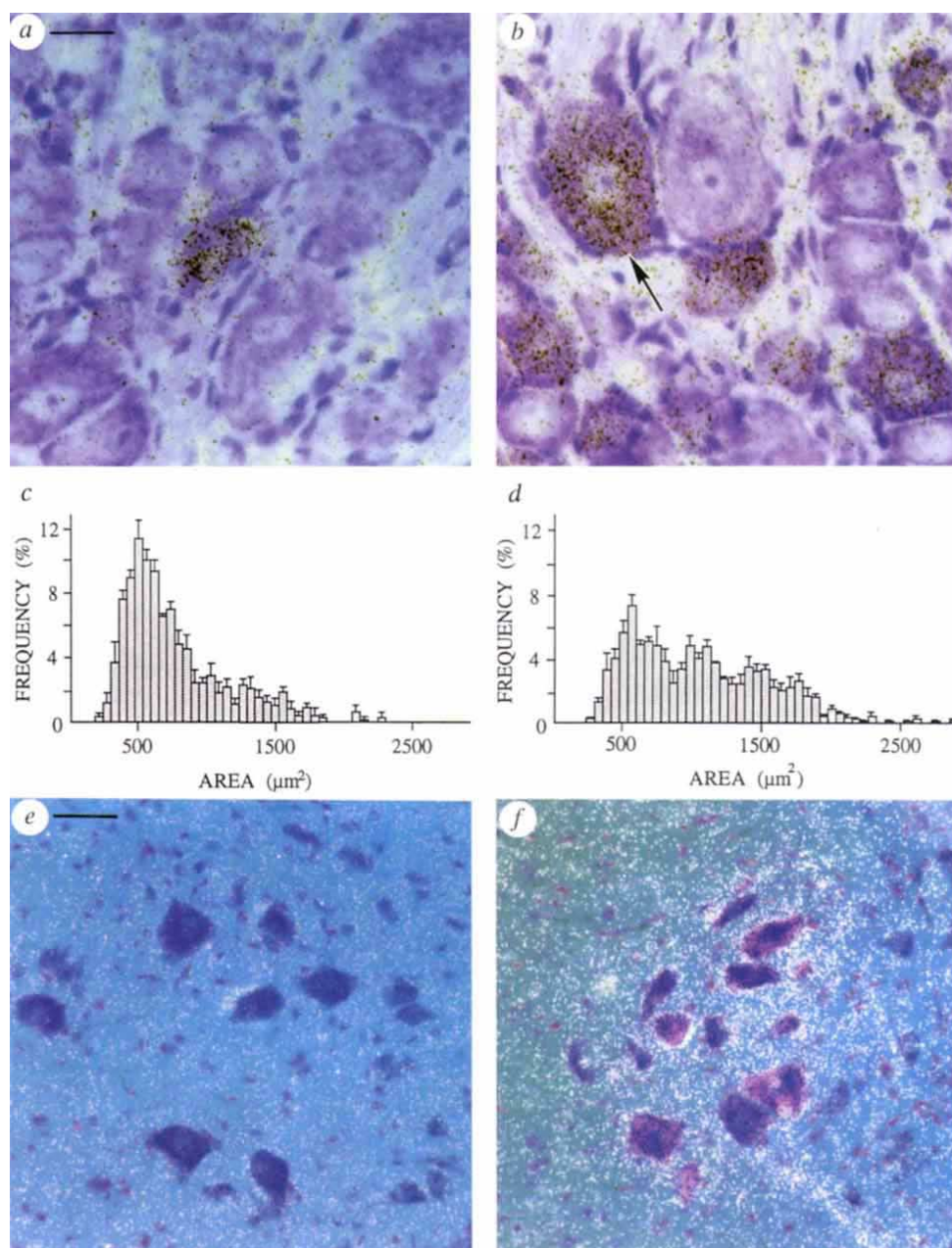
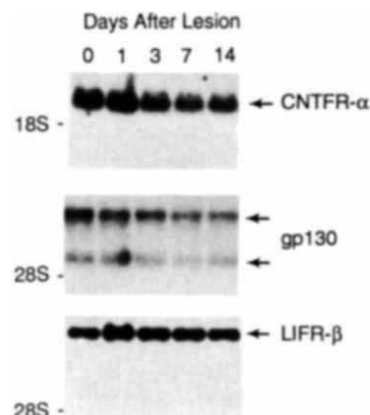


FIG. 3 Expression of CNTFR- α (top), gp130 (middle) and LIFR- β (bottom) mRNA in lumbar DRG after sciatic nerve crush lesion.

METHOD. At various times (0, 1, 3, 7, 14 days) after a crush lesion (see legend to Fig. 1) DRG were removed from 6–10 animals per group, homogenized in 4 M guanidine/2% β -mercaptoethanol and total RNA was extracted as in ref. 27. Total RNA ($10\ \mu\text{g}$) from each sample was separated on 1% agarose/formamide gels, transferred to nylon membranes (Pharmacia), and membranes were prehybridized in $2 \times$ Denhardt's reagent, 0.5% SDS, $100\ \mu\text{g ml}^{-1}$ salmon sperm DNA, 50% formamide in $6 \times$ SSPE buffer. Membranes were then hybridized to cDNA probes for rat CNTFR- α (400 bp), rat LIFR- β (540 bp) or human gp130 (2.0 kb)⁷. 18S and 28S ribosomal markers are indicated to the left.



myelin or increased axonal surface area due to sprouting. Third, non-neuronal cells in the distal nerve and denervated muscle may help present CNTF to axons through the expression of cell surface or soluble receptors^{9,12}. A fourth possibility is that endogenous CNTF affects retrograde transport of ¹²⁵I-CNTF (see below).

CNTF levels are extremely high in Schwann cells of intact sciatic nerve but are greatly decreased distal to the site of nerve injury 4–7 days post-lesion^{13–15}. It was thus determined if lesion-induced depletion of endogenous CNTF was the cause of increased ¹²⁵I-CNTF transport after nerve crush. At any time between 1 and 7 days after lesion there was a similar increase in retrograde transport to sensory neurons (Fig. 4b, c, d), whereas no increase was observed when ¹²⁵I-CNTF was injected immediately (1–2 min) after nerve crush (data not shown). As CNTF levels do not decrease in distal nerve until 4–7 days after transection^{13–15}, it is unlikely that CNTF depletion accounts for the elevated ¹²⁵I-CNTF transport observed 1 day after nerve crush. This was tested directly by injecting ¹²⁵I-CNTF proximal to a crush at a point where Schwann cells display normal CNTF levels. The sciatic nerve was crushed at the knee and ¹²⁵I-CNTF was injected 1.5 cm proximally 1 or 7 days later (Fig. 4a). In this paradigm, retrograde transport was increased twofold at all times compared to unlesioned animals (Fig. 4e), suggesting that elevated CNTF retrograde transport following nerve injury is not affected by endogenous CNTF levels. CNTF accumulation

in DRG appeared to be more rapid after injection directly into the crush site. This observation supports the possibility that axons may have greater access to CNTF when injections are made directly into damaged nerves. Nonetheless, enhanced CNTF retrograde transport following a lesion is a receptor-mediated event, and was blocked by excess unlabelled CNTF in all lesion paradigms.

This study represents the first demonstration of retrograde axonal transport of CNTF to sensory and motor neurons, that are known to respond to CNTF *in vitro*^{1,2}. This is in agreement with previous studies showing that populations of neurons responding to neurotrophins and other putative neurotrophic factors *in vitro* accumulate the same factors by retrograde transport *in vivo*^{16–19} and supports the hypothesis that retrograde transport is a common mechanism whereby trophic factors presented at distant axon terminals can elicit signals in neuronal cell bodies.

A novel feature of the present study is the finding that prior nerve injury leads to dramatically increased transport of CNTF. It has been suggested that CNTF is released by Schwann cells as a consequence of neural injury^{3,14}. In support of this, CNTF-like activity is present in the medium of Schwann cell cultures²⁰ and, although the mechanism is currently unknown, it remains possible that released CNTF becomes available to regenerating neurons shortly after injury¹⁴. The temporal and spatial availability of CNTF in injured neural tissue, coordinated with increased capacity for retrograde transport, may be integral to the regenerative effort of neurons after interruption of their axons.

Retrograde transport is significant in the pharmacological delivery of neurotrophic factors to the central nervous system. In animal studies, retrograde transport may provide a means by which CNTF gains access to central neurons after peripheral administration. For example, axotomy-induced death of neonatal facial motor neurons was prevented by application of CNTF to the stump of the cut nerve²¹ and recent studies have shown that systemic administration of CNTF attenuates neuromuscular deficits in mouse models of motor neuron degeneration, such as *pnn/pnn*, *Mnd* and *wobbler*^{22–24}. Furthermore, damage to peripheral neurons resulting from trauma, toxic or diabetic neuropathies and disease may increase axonal retrograde transport, and thus efficacy, of exogenously administered neurotrophic factors. □

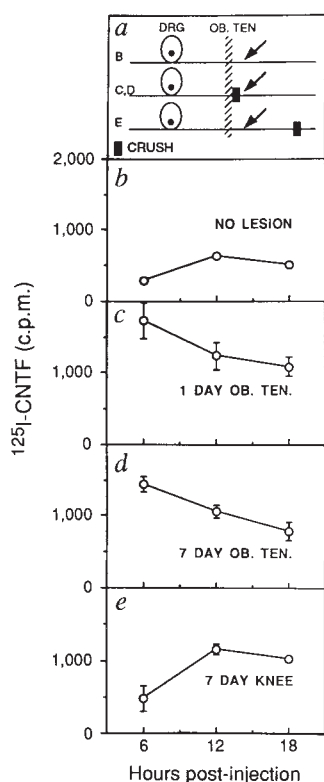


FIG. 4 Role of endogenous CNTF in elevated ¹²⁵I-CNTF retrograde transport after lesion. The time course of ¹²⁵I-CNTF retrograde transport to lumbar DRG was assessed in normal animals or after various types of sciatic nerve lesion, as depicted in a. Hatched area indicates where the sciatic nerve overlies the tendon of the obturator internus muscle (OB. TEN.) and solid bars indicate the crush sites. Intraneural injections of ¹²⁵I-CNTF were always made at the OB. TEN. (arrows). b, No lesion, injection at OB. TEN.; c, nerve crushed at OB. TEN., injection at OB. TEN. 1 day later; d, nerve crushed at OB. TEN., injection at OB. TEN. 7 days later; e, nerve crushed at KNEE (1.5 cm distal to OB. TEN.), injection at OB. TEN. 7 days later. c.p.m. from right L4 + L5 DRG were pooled and represent mean $\pm$ s.d., $n=3$. Absence of error bar indicates the deviation did not exceed the symbol size.

Received 19 April; accepted 28 June 1993.

- Barbin, G., Manthorpe, M. & Varon, S. *J. Neurochem.* **43**, 1468–1478 (1984).
- Arakawa, Y., Sendtner, M. & Thoenen, H. *J. Neurosci.* **10**, 3507–3515 (1990).
- Thoenen, H. *Trends Neurosci.* **14**, 165–170 (1991).
- Oppenheim, R. W., Prevette, D., Qin-Wei, Y., Collins, F. & MacDonald, J. *Science* **251**, 1616–1618 (1991).
- Ip, N. Y. *et al. Cell* **69**, 1121–1132 (1992).
- Stöckli, K. A. *et al. Nature* **342**, 920–923 (1989).
- Ip, N. Y. *et al. Neuron* **10**, 89–102 (1993).
- Davis, S. *et al. Science* **253**, 59–63 (1991).
- Davis, S. *et al. Science* **259**, 1736–1739 (1993).
- Gearing, D. P. *et al. Science* **255**, 1434–1437 (1992).
- Taga, T. *et al. Cell* **58**, 573–581 (1989).
- Johnson, E. M., Taniuchi, M. & DiStefano, P. S. *Trends Neurosci.* **7**, 299–304 (1988).
- Friedman, B. *et al. Neuron* **9**, 295–305 (1992).
- Sendtner, M., Stöckli, K. A. & Thoenen, H. *J. Cell Biol.* **118**, 139–148 (1992).
- Seniuk, N., Altare, M., Dunn, R. & Richardson, P. M. *Brain Res.* **572**, 300–302 (1992).
- Hendry, I. A., Stöckli, K., Thoenen, H. & Iversen, L. L. *Brain Res.* **68**, 103–121 (1974).
- DiStefano, P. S. *et al. Neuron* **8**, 983–993 (1992).
- Ferguson, I. A., Schweitzer, J. B., Bartlett, P. F. & Johnson, E. M. *J. comp. Neurol.* **313**, 680–692 (1991).
- Hansson, H.-A., Rozell, B. & Skottner, A. *Cell Tiss. Res.* **247**, 241–247 (1987).
- Meyer, M., Matsuoaka, I., Wetmore, C., Olson, L. & Thoenen, H. *J. Cell Biol.* **119**, 45–54 (1992).
- Sendtner, M., Kreutzberg, G. W. & Thoenen, H. *Nature* **345**, 440–441 (1990).
- Sendtner, M. *et al. Nature* **358**, 502–504 (1992).
- Helgren, M. *et al. Soc. Neurosci. Abstr.* **18**, 618 (1992).
- Holmlund, T. H. *et al. Neurology* **42** (suppl. 3), 369 (1992).
- Masiakowski, P. *et al. J. Neurochem.* **57**, 1003–1012 (1991).
- Marchalonis, J. J. *Biochem. J.* **113**, 299–305 (1969).
- Chomczynski, P. & Sacchi, N. *Analyt. Biochem.* **162**, 156–159 (1987).

ACKNOWLEDGEMENTS. We thank N. Stambler for statistical analysis, A. Acheson and members of the Regeneron bioassay group for analysis of iodinated CNTF, and the Regeneron community for critical discussions and assistance. This work is dedicated to the memory of Professor Victor DiStefano.

Preferential inhibition of ω -conotoxin-sensitive presynaptic Ca^{2+} channels by adenosine autoreceptors

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ADENOSINE is a potent modulator of transmitter release at a variety of synapses. The adenosine A1 receptor is assumed to reside in presynaptic terminals and to function as a negative autoreceptor¹. How adenosine reduces transmitter release is uncertain²; it may reduce the calcium influx during nerve terminal depolarization by either activating K^+ currents^{3,4} or inhibiting Ca^{2+} currents^{5,6}, although other mechanisms have been proposed⁷⁻⁹. We have directly measured intracellular Ca^{2+} concentrations of giant presynaptic terminals in the chick ciliary ganglion. We report here that adenosine inhibited the nerve-evoked Ca^{2+} influx in the terminal by activating A1 receptors. Reduced Ca^{2+} influx was due largely to inhibition of ω -conotoxin GVIA-sensitive Ca^{2+} channels in the presynaptic terminal.

Large calyciform terminals of the chick ciliary ganglion were labelled by dextran (M_r 10K)-conjugated Fura-2 (Fura-dextran) transported anterogradely by axonal flow (Fig. 1a). Because of its large size, Fura-dextran was confined to the presynaptic axons and their terminals. A photomultiplier was focused on 1-3 terminals and the fluorescent signals were monitored by alternating the excitation wavelength between 340 and 380 nm, and the intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_{\text{pre}}$) was quantified by the ratio of their fluorescence intensities (Fig. 1b). With the standard extracellular solution containing 5 mM Ca^{2+} and 1 mM Mg^{2+} , the resting $[\text{Ca}^{2+}]_{\text{pre}}$ was 20–60 nM ($n=10$); $[\text{Ca}^{2+}]_{\text{pre}}$ increased by 10–20 nM ($\Delta[\text{Ca}^{2+}]_{\text{pre}}$) following a single nerve stimulus. Accumulation of $\Delta[\text{Ca}^{2+}]_{\text{pre}}$ was observed at short intervals (Fig. 1b). The $[\text{Ca}^{2+}]_{\text{pre}}$ increased with a short

latency, peaked within 50–100 ms and decayed rapidly (Fig. 1c). The $\Delta[\text{Ca}^{2+}]_{\text{pre}}$ was not detected in Ca^{2+} -free solution containing 1 mM EGTA (Fig. 1d) or when Ca^{2+} channels were blocked by Cd^{2+} (0.5 mM).

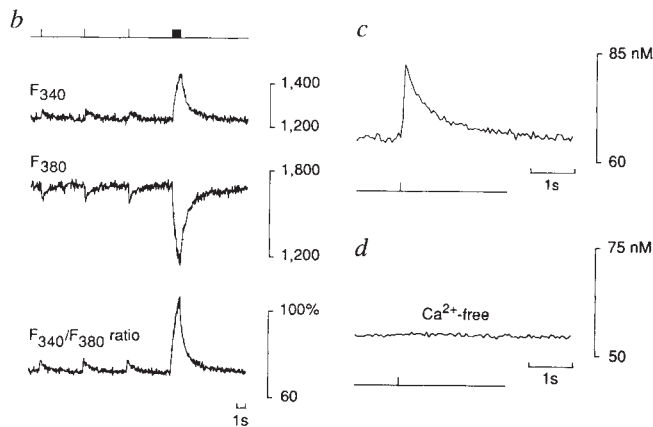
As reported previously¹⁰, the excitatory synaptic current (e.p.s.c.) was reduced by adenosine (Fig. 2a). In the presence of 100 μM adenosine, the e.p.s.c. amplitude was $38 \pm 14\%$ of the control (mean and s.d., $n=6$; Fig. 3a). The e.p.s.c. was also inhibited by ATP; this is expected because ATP is generally a cotransmitter in cholinergic neurons and is rapidly hydrolysed to adenosine¹¹. The adenosine A1 receptor-specific antagonist 8-cyclopentyltheophylline (CPT, 1 μM), which is widely used for neurons⁶, blocked the effects of adenosine (Fig. 2a) or ATP. Similar results were observed for another A1-antagonist, 8-phenyltheophylline (8-PT, 10 μM). It is thus likely that inhibition of e.p.s.c. by ATP/adenosine is mediated through the A1 adenosine receptors¹⁰.

How might adenosine reduce the e.p.s.c.? Adenosine (100 μM) also suppressed $\Delta[\text{Ca}^{2+}]_{\text{pre}}$ to $73 \pm 5\%$ ($n=10$; Fig. 3a). Again, ATP (100 μM) mimicked the effect of adenosine, reducing it to $75 \pm 5\%$ ($n=6$). The effective dose of ATP on $\Delta[\text{Ca}^{2+}]_{\text{pre}}$ was as low as 10 μM . These inhibitory effects of adenosine or ATP were antagonized by CPT (Fig. 2b) or 8-PT. The effect of adenosine was completely reversible.

The suppression of $\Delta[\text{Ca}^{2+}]_{\text{pre}}$ by adenosine might be attributed either to reduced Ca^{2+} influx or to enhanced intracellular Ca^{2+} sequestration. Adenosine has been suggested to decrease the resting $[\text{Ca}^{2+}]_{\text{pre}}$, thereby interfering with transmitter release^{12,13}. In our experiments, however, the resting $[\text{Ca}^{2+}]_{\text{pre}}$ was not affected by adenosine ($n=3$). When we replaced extracellular Ca^{2+} with Ba^{2+} , nerve stimulation again increased the fluorescence ratio, indicating a change of intracellular Ba^{2+} concentration (Fig. 2c; $[\text{Ba}^{2+}]_{\text{pre}}$). It is almost certain that this increase of $[\text{Ba}^{2+}]_{\text{pre}}$ ($\Delta[\text{Ba}^{2+}]_{\text{pre}}$) represents Ba^{2+} influx through Ca^{2+} channels^{14,15}; the $\Delta[\text{Ba}^{2+}]_{\text{pre}}$ was estimated to be 326 ± 153 nM ($n=5$), with a K_D of 1,360 nM¹⁵. Adenosine reduced $\Delta[\text{Ba}^{2+}]_{\text{pre}}$ by 32–40% ($n=5$; Fig. 2d); hence, adenosine must inhibit Ba^{2+} (Ca^{2+}) influx. But the $\Delta[\text{Ba}^{2+}]_{\text{pre}}$ declined to basal level very slowly (Fig. 2c) with a time constant of 50–60 s (data not shown). The high $\Delta[\text{Ba}^{2+}]_{\text{pre}}$ and its slow decline indi-

FIG. 1 Measurement of the intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_{\text{pre}}$) of the giant presynaptic terminals of chick ciliary ganglion. a, A large calyciform terminal as well as its axon (asterisk) labelled by dextran-conjugated Fura-2 (Fura-dextran). Scale bar, 10 μm . b, Top, stimulus applied to the presynaptic oculomotor nerve, indicating 3 single stimuli followed by a 10 Hz train for 100 ms. Middle two traces, the change of fluorescence of a single nerve terminal measured by excitation wavelengths of 340 and 380 nm. Bottom, the ratio of their fluorescence intensities. c, Stimulation-triggered averaging of the $[\text{Ca}^{2+}]_{\text{pre}}$ response to single presynaptic stimuli. From the same terminal as shown in b. d, The averaged $[\text{Ca}^{2+}]_{\text{pre}}$ response of the same terminal in a Ca^{2+} -free solution containing 1 mM EGTA.

METHODS. Chick embryos (day 14) were decapitated, the presynaptic oculomotor nerve was cut at its exit from the orbital bone in a Ca^{2+} -free solution containing 1 mM EGTA and 10 mM MgCl_2 . Crystals of Fura-dextran (Molecular Probes) were applied to the cut end of the distal stump. After 30 min of incubation at 10 $^{\circ}\text{C}$, the ganglion was superfused with the oxygenated standard solution (mM): NaCl 135, KOH 5, CaCl_2 5, MgCl_2 1, HEPES 10, mannitol 1, glucose 11, pH 7.4 adjusted with HCl. After incubating at 36 $^{\circ}\text{C}$ for 1.5 h, the collagenous envelope of the ganglion was removed by focal application of a mixture of collagenase and thermolysin through a pipette with a 40- μm tip diameter for 15 min



at 19–21 $^{\circ}\text{C}$ ¹⁸. A conventional epifluorescence system equipped with a water-immersion objective (Olympus $\times 40$ NA 0.7) and Xenon lamp (150 W) was used. The methods used for measuring fluorescence intensity with a photomultiplier have been described¹⁹. The signal was integrated for 50 ms and sampled at 20 Hz. The $[\text{Ca}^{2+}]_{\text{pre}}$ was calculated from the ratio of fluorescence intensity at wavelengths of 340 and 380 nm²⁰ using a K_D of 350 nM²¹. The minimum ratio and the maximum fluorescence at 380 nm were measured in a Ca^{2+} -free solution containing 5 mM BAPTA and 0.1 mM ionomycin (Calbiochem). Thereafter the solution was changed to that containing 10 mM CaCl_2 , and the maximum ratio and the minimum fluorescence at 380 nm were measured. To reduce the signal-to-noise ratio, 60 records were averaged, using each stimulating pulse as a trigger.

cate that the rate of sequestering intracellular Ba^{2+} is much slower than that for Ca^{2+} . This slow decline of $\Delta[\text{Ba}^{2+}]_{\text{pre}}$ remained unchanged by adenosine (Fig. 2c, lower trace). In two experiments, the presynaptic Ba^{2+} currents were measured under whole-cell voltage clamp (Fig. 2e). The effects of adenosine were time-dependent and the reductions of Ba^{2+} currents by 16–17% were observed at 4 ms.

The chick ciliary calyx has two distinct subpopulations of Ca^{2+} channel¹⁶. The major type was sensitive to ω -conotoxin GVIA (ω -CgTx) but insensitive to dihydropyridines (DHPs). The other type was resistant to both ω -CgTx and DHPs. Might one of the two types of Ca^{2+} channel be affected by adenosine preferentially? If so, the extent of inhibition of Ca^{2+} -influx by adenosine would differ before and after ω -CgTx application. One obstacle to this test is that a large part of the Ca^{2+} influx is blocked by ω -CgTx, so that the effect of adenosine on the small ω -CgTx-resistant Ca^{2+} influx is difficult to evaluate. For this reason, the Ca^{2+} influx was augmented by applying 4-

aminopyridine (4-AP, 0.4 mM), which prolonged the presynaptic action potential duration without affecting the resting potential (H.Y. and N.C., manuscript in preparation). The $\Delta[\text{Ca}^{2+}]_{\text{pre}}$ observed by a single presynaptic stimulation under this condition was 25–90 nM ($n=7$; Fig. 4a), about 4.5-fold greater than the value in the standard solution without 4-AP. Adenosine (100 μM) reduced the $\Delta[\text{Ca}^{2+}]_{\text{pre}}$ to $78 \pm 6\%$ (range, 67–85%; $n=7$), which is comparable to that in the standard solution. When treated with ω -CgTx (10 μM), the $\Delta[\text{Ca}^{2+}]_{\text{pre}}$ was irreversibly reduced by about 80% (Fig. 4b, c), being 7–18 nM ($n=7$). The ω -CgTx-resistant component of the $\Delta[\text{Ca}^{2+}]_{\text{pre}}$ was virtually unaffected by adenosine (100 μM), as shown in Fig. 4b and c. In seven experiments, adenosine never reduced the $\Delta[\text{Ca}^{2+}]_{\text{pre}}$ by more than 10% after ω -CgTx application. We also increased external Ca^{2+} to 10 mM to enhance $\Delta[\text{Ca}^{2+}]_{\text{pre}}$ in the absence of 4-AP. Again, adenosine did not reduce the ω -CgTx-resistant component of $\Delta[\text{Ca}^{2+}]_{\text{pre}}$ by more than 10% in four experiments. These effects of adenosine on $\Delta[\text{Ca}^{2+}]_{\text{pre}}$ and e.p.s.c.s (Fig. 3a)

FIG. 2 Suppression of Ca^{2+} influx by adenosine. a, Sample records of the excitatory synaptic current (e.p.s.c.) in a postsynaptic ciliary neuron (top). The inhibitory effect of adenosine (100 μM) was reduced by the A_1 -adenosine receptor specific antagonist, 8-cyclopentyltheophylline (CPT, 1 μM). Bottom, changes in the height of e.p.s.c. of the same cell. b, Top, sample records of $[\text{Ca}^{2+}]_{\text{pre}}$ in response to single presynaptic stimuli. The inhibitory effect of adenosine (100 μM) was reversed by CPT (1 μM). Bottom, the increase of $[\text{Ca}^{2+}]_{\text{pre}}$ ($\Delta[\text{Ca}^{2+}]_{\text{pre}}$) was plotted against time in the same experiment. c, The response of intracellular Ba^{2+} in a presynaptic terminal ($[\text{Ba}^{2+}]_{\text{pre}}$) to a single presynaptic stimulus in the absence (top) or presence of 100 μM adenosine (bottom). The $[\text{Ba}^{2+}]_{\text{pre}}$ decayed to the basal level with a time constant of 48 s (data not shown). d, Changes in the increase of $[\text{Ba}^{2+}]_{\text{pre}}$ ($\Delta[\text{Ba}^{2+}]_{\text{pre}}$) in response to single presynaptic stimuli of the same terminal as in c. e, Suppression of the presynaptic Ba^{2+} current by adenosine (100 μM). The Ba^{2+} currents were elicited by pulses to -10 mV from a holding potential of -80 mV (top). METHODS. A conventional whole-cell patch clamp recording²² was made from a postsynaptic ciliary neuron. The pipette solution contained (in mM), CsCl 140, BAPTA 5, HEPES 10, Mg-ATP 5, pH 7.4, adjusted with NaOH. The results were not affected by removal of ATP. The presynaptic nerve was stimulated at 0.5 Hz, and every 10 e.p.s.c.s were averaged. For the measurement of $[\text{Ca}^{2+}]_{\text{pre}}$, 60 records were averaged. The $[\text{Ba}^{2+}]_{\text{pre}}$ was measured in a solution containing 5 mM Ba^{2+} without Ca^{2+} or Mg^{2+} . The presynaptic nerve was stimulated every 2 min. The

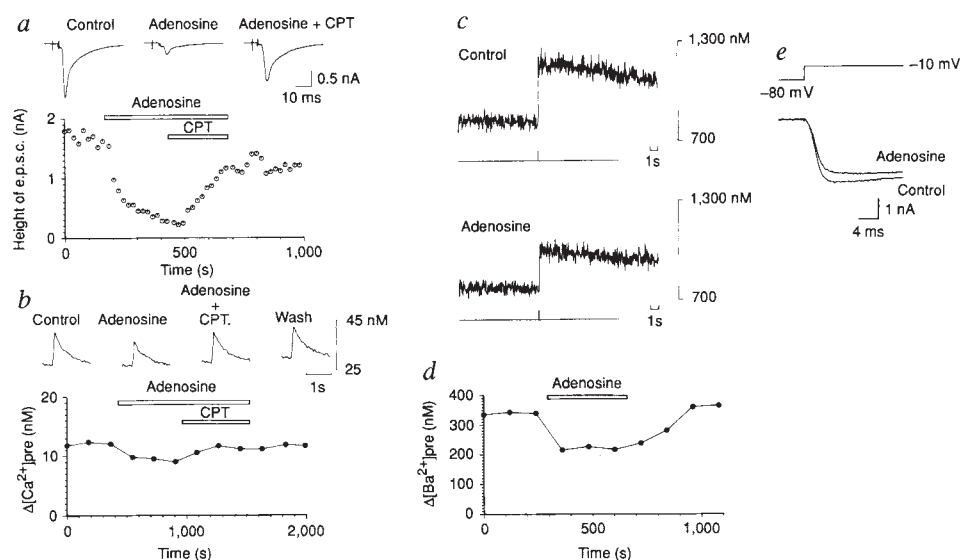
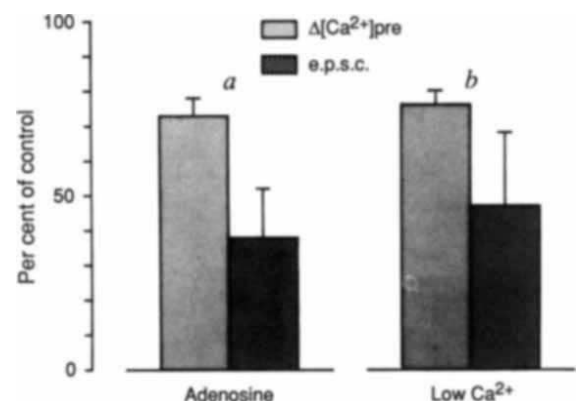


FIG. 3 Comparison of the effects of 100 μM adenosine (a) with those of lowering extracellular Ca^{2+} from 5 mM to 2.5 mM (b) on both the $\Delta[\text{Ca}^{2+}]_{\text{pre}}$ and the e.p.s.c. Each bar indicates s.d. of the mean.



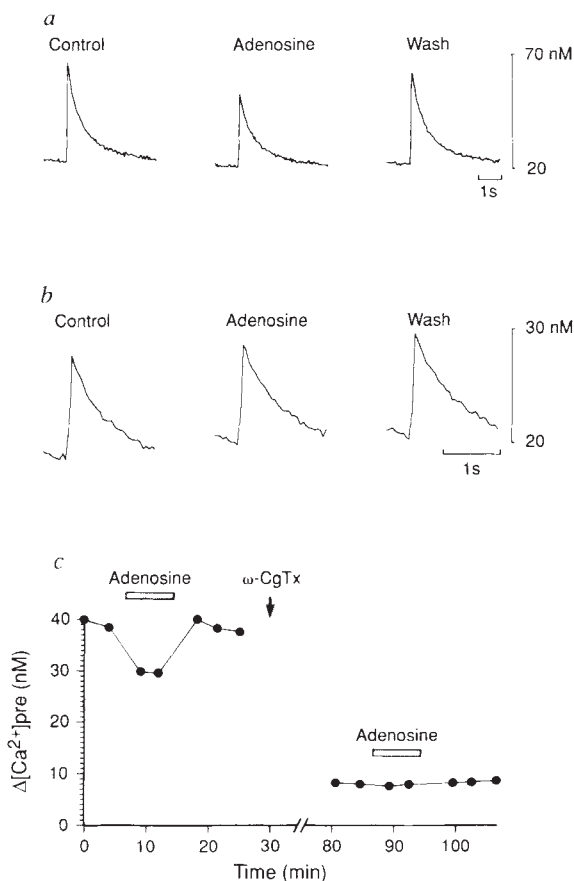


FIG. 4 Preferential inhibition of ω -conotoxin GVIA(ω -CgTx)-sensitive Ca^{2+} influx by adenosine. *a*, The effect of adenosine (100 μM) on $\Delta[\text{Ca}^{2+}]_{\text{pre}}$ evoked by a single presynaptic stimulus in a solution containing 0.4 mM 4-aminopyridine (4-AP). To minimize the inactivation of ω -CgTx-resistant Ca^{2+} channels, the external K^+ was reduced to 1 mM; the resting potential of presynaptic terminals observed in this solution was about -70 mV (H.Y. and N.C., manuscript in preparation). The presynaptic nerve was stimulated at 0.2 Hz and 12 records were averaged. *b*, The effect of adenosine (100 μM) on the same terminal as in *a*, after treating with 10 μM ω -CgTx for 30 min. The presynaptic nerve was stimulated at 0.5 Hz and 60 records were averaged. *c*, Changes of $\Delta[\text{Ca}^{2+}]_{\text{pre}}$ in the same terminal as for *a* and *b*.

could be reproduced surprisingly well by reducing the external Ca^{2+} concentration. When the external Ca^{2+} was halved from 5 mM to 2.5 mM, the $\Delta[\text{Ca}^{2+}]_{\text{pre}}$ decreased to $76 \pm 4\%$ ($n=7$), and the e.p.s.c. to $47 \pm 21\%$ ($n=6$; Fig. 3*b*). The greater effect on the e.p.s.c. can be accounted for by the nonlinear relation between the Ca^{2+} influx and the transmitter release¹⁷. Thus, the action of adenosine on the synaptic transmission can be attributed mostly to suppression of the presynaptic Ca^{2+} channels, although the mechanisms downstream to Ca^{2+} influx might be involved⁷⁻⁹. As noted, however, adenosine preferentially blocked the ω -CgTx-sensitive Ca^{2+} channels. This may reflect that the ω -CgTx-sensitive Ca^{2+} channels and the adenosine A1 receptors are closely located in the presynaptic terminal. Alternatively, the ω -CgTx-resistant Ca^{2+} channels would lack the molecular domain to respond to the A1 receptor activation. The results in Fig. 4 also suggest that the modulation of K^+ channels may not be involved in the adenosine-dependent inhibition of transmitter release. □

- Trussell, L. O. & Jackson, M. B. *Proc. natn. Acad. Sci. U.S.A.* **82**, 4857–4861 (1985).
- Gerber, U., Greene, R. W., Haas, H. L. & Stevens, D. R. *J. Physiol., Lond.* **417**, 567–578 (1989).
- Hamilton, B. P. & Smith, D. O. *J. Physiol., Lond.* **432**, 327–341 (1991).
- Scholz, K. P. & Miller, R. J. *J. Physiol., Lond.* **435**, 373–393 (1991).
- Silinsky, E. M. *Trends pharmac. Sci.* **7**, 180–185 (1986).
- Scholz, K. P. & Miller, R. J. *Neuron* **8**, 1139–1150 (1992).
- Silinsky, E. M. & Solsona, C. S. *J. Physiol., Lond.* **457**, 315–328 (1992).
- Bennett, M. R. & Ho, S. J. *J. Physiol., Lond.* **440**, 513–527 (1991).
- Snyder, S. H. *Rev. Neurosci.* **8**, 103–124 (1985).
- Branisteau, D. D., Haulica, I. D., Proca, B. & Nhue, B. G. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **308**, 273–279 (1979).
- Scanziani, M., Capogna, M., Gähwiler, B. G. & Thompson, S. M. *Neuron* **9**, 919–927 (1992).
- Schilling, W. P., Rajan, I. & Strobl-Jager, E. *J. biol. Chem.* **264**, 12838–12848 (1989).
- Kwan, C.-Y. & Putney, J. W. Jr *J. biol. Chem.* **265**, 678–684 (1990).
- Yawo, H. & Momiyama, A. J. *J. Physiol., Lond.* **460**, 153–172 (1993).
- Dodge, F. A. Jr & Rahamimoff, R. *J. Physiol., Lond.* **193**, 419–432 (1967).
- Yawo, H. *J. Physiol., Lond.* **417**, 307–322 (1989).
- Shimohara, T., Icard-Liepkalins, C., Ohmori, H. & Shigemoto, T. *Brain Res.* **524**, 219–224 (1990).
- Grynkiewicz, G., Poenie, M. & Tsien, R. Y. *J. biol. Chem.* **260**, 3440–3450 (1985).
- Haugland, R. P. in *Handbook of Fluorescent Probes and Research Chemicals* 5th edn 114 (Molecular Probes Inc., Eugene, 1992).
- Hamill, O. P., Marty, A., Neher, E., Sakmann, B. & Sigworth, F. J. *Pflügers Arch.* **391**, 85–100 (1981).

ACKNOWLEDGEMENTS. We thank M. Kuno, Y. Okada and H. Ohmori for comments on the manuscript and K. Furuya for providing software for measuring intracellular calcium (MiCa). This work was supported by Grants-in-Aid from the Ministry of Education, Science and Culture of Japan and the Uehara Memorial Foundation.

G-protein modulation of ion permeation through N-type calcium channels

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N-TYPE calcium channels in cell membranes are inhibited by neurotransmitters such as noradrenaline¹⁻⁴, luteinizing hormone-releasing hormone (LHRH)⁵⁻⁷, γ -aminobutyric acid (GABA)^{8,9} and glutamate^{10,11}. Although GTP-binding proteins (G proteins) are probably the common mediators of such inhibition^{6,11-14}, it is unclear exactly how G proteins alter the operation of the channel. Various experiments have shown changes in channel gating^{2-6,15,16}. Here we show that inward current carried by Na^+ through N-type channels was far less inhibited by LHRH or by internal GTP- γS than was current carried by Ba^{2+} . With external Ba^{2+} and internal Cs^+ , LHRH inhibited the Ba^{2+} -carried inward limb of the instantaneous current-voltage curve much more than the Cs^+ -carried outward limb. Noise analysis showed that LHRH or GTP- γS decrease single-channel current carried by Ba^{2+} . These results suggest that alteration of the ion permeation pathway contributes significantly to G-protein inhibition of N-type Ca^{2+} channels.

Neurotransmitters acting on N-type Ca^{2+} channels often produce dramatic inhibition at moderate depolarizations, where inward current is carried by external Ca^{2+} or Ba^{2+} , but have much smaller effects at strong depolarizations, with outward currents carried by internal monovalent ions. Such findings have so far been ascribed solely to changes in channel gating^{3-6,15}. To test whether the species of permeating ion is relevant (a possibility previously raised for different divalent ions¹⁷), external Ba^{2+} was replaced with Na^+ and the inhibition of inward monovalent N-type channel currents was measured. The effect of LHRH was remarkably smaller in Na^+ than in Ba^{2+} (Fig. 1*a, b*). The LHRH inhibition of Na^+ -carried current was moderate in cells with strong inhibition of Ba^{2+} current and almost non-existent in cells with moderate inhibition of Ba^{2+} current (Fig. 1*c*).

Similar results were obtained with GTP- γS in the internal solution to activate G proteins directly and inhibit N-type channels. Here, strong depolarizing prepulses can remove the

Received 26 February; accepted 24 June 1993.

- Nicoll, R. A., Malenka, R. & Kauer, J. A. *Physiol. Rev.* **70**, 513–565 (1990).
- Greene, R. W. & Haas, H. L. *Progr. Neurobiol.* **36**, 329–341 (1991).

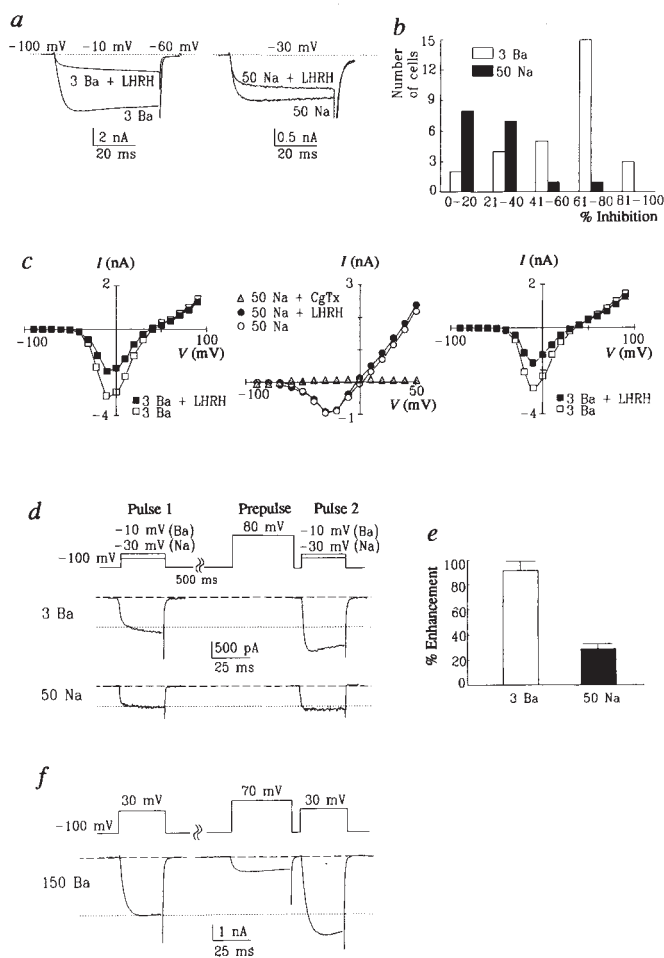


FIG. 1 LHRH and GTP- γ S inhibition of Ba^{2+} and Na^{+} currents through N-type channels. **a**, LHRH (1 μM) inhibition of N-type current carried by 3 mM Ba^{2+} or 50 mM Na^{+} . **b**, Distribution of percentage inhibition by LHRH for Ba^{2+} or Na^{+} currents. Average inhibition was $61 \pm 4\%$ ($n=29$) for Ba^{2+} and $20 \pm 5\%$ ($n=17$) for Na^{+} . LHRH at 10 μM had no more effect than 1 μM on monovalent currents. **c**, Inhibition of Ba^{2+} and Na^{+} currents in the same cell. Na^{+} currents were unaffected, whereas Ba^{2+} currents (recorded before and after the Na^{+} current measurements) were inhibited by $\sim 45\%$. **d**, Prepulse enhancement of currents carried by Ba^{2+} or Na^{+} with 0.3 mM GTP- γ in the internal solution. A 40-ms prepulse to +80 mV enhanced the current during a test pulse (-30 mV for Na^{+} , -10 mV for Ba^{2+}). **e**, In 8 cells studied with both, the enhancement (the test pulse currents were measured at 5 ms) was $92 \pm 9\%$ for 3 mM Ba^{2+} and $31 \pm 5\%$ for 50 mM Na^{+} . **f**, Prepulse enhancement of current carried by 150 mM Ba^{2+} showing that enhancement does not require the channel pore to be cleared of divalent ions during the prepulse. In 8 cells studied with both, prepulse enhancement was less for 150 mM Ba^{2+} ($62 \pm 6\%$) than for 3 mM Ba^{2+} ($88 \pm 6\%$).

METHODS. Currents through N-type calcium channels in bullfrog sympathetic neurons were recorded as previously described⁵. Except where noted, the internal solution contained (in mM): 150 CsCl, 9 EGTA, 9 HEPES, 4.5 MgCl_2 , 14 creatine phosphate (Tris salt), 4 Mg-ATP , 0.3 GTP (Tris salt); pH adjusted to 7.4 with CsOH. The usual external solution contained (in mM): 3 BaCl_2 , 150 tetraethylammonium chloride (TEA-Cl), 10 HEPES, pH adjusted to 7.4 with TEA-hydroxide. As indicated, the 3 BaCl_2 + 150 TEA-Cl was replaced by 50 NaCl + 100 TEA-Cl + 2 H-EDTA or by 150 BaCl_2 . All solutions contained 5 μM tetrodotoxin and also 3 μM nimodipine to block L-type Ca^{2+} channels; with these conditions, inward currents with Na^{+} as well as Ba^{2+} were virtually completely blocked by 3 μM ω -conotoxin (CgTx). In all cases (except in **c**) where outward currents are shown or measured, CgTx-insensitive current was subtracted. Statistics are given as mean $\pm$ standard error of mean. Terminology: for ease of expression, we have used 'G-protein binding' to refer to the G-protein-mediated modulation of the channels, but no conclusions about permeability changes would be altered if the modulatory effects were exerted less directly by the G proteins.

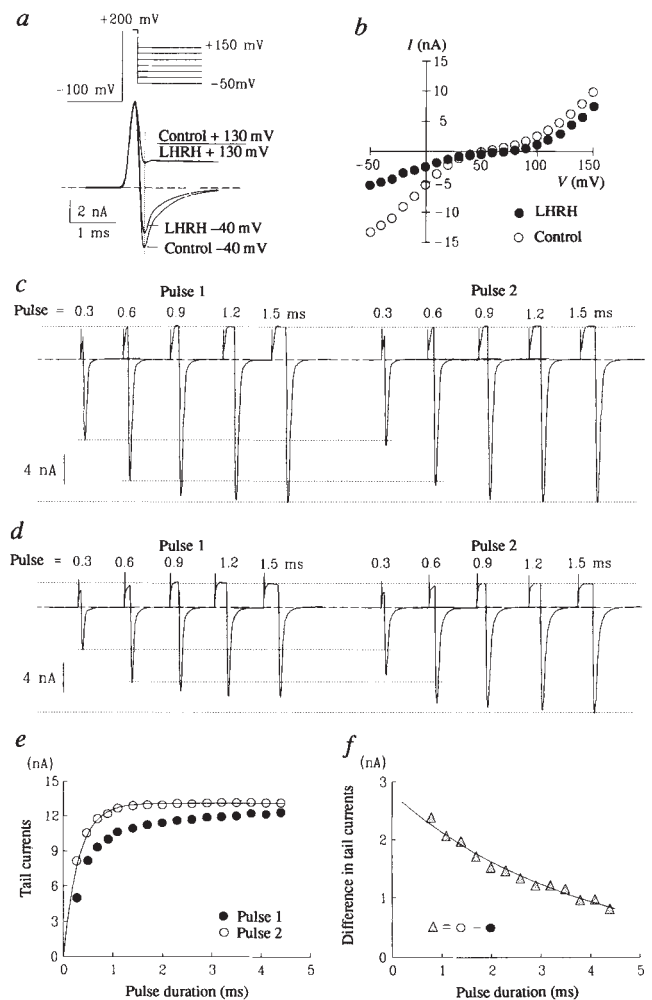


FIG. 2 LHRH and GTP- γ S effects on the instantaneous current-voltage (I - V) relationship. In all cases, currents through CgTx-sensitive N-type channels were isolated by subtracting CgTx-insensitive current from every sweep. **a**, LHRH (1 μM) inhibition of inward Ba^{2+} (3 mM) tail current but not outward Cs^{+} tail current following a short pulse (0.3 ms, 200 mV) to activate channels. Voltage errors from uncompensated series resistance were less than 2 mV. **b**, Instantaneous tail I - V curve from another cell (with larger inhibition of inward current and slight inhibition of outward current) using the same protocol. Currents were filtered at 10 kHz (4-pole Bessel) and measured at the time of peak filtered tail current ~ 300 μs after the voltage change. Voltage errors due to uncompensated series resistance were < 3 mV. **c**, **d**, Prepulse effects on outward Cs^{+} currents and inward Ba^{2+} currents in a cell with internal 0.3 mM GTP- γ S instead of GTP. The voltage protocol is similar to that in Fig. 1d, with a 40-ms prepulse to +110 mV between two test pulses. The two test pulses (pulse 1 and 2) were to +110 mV (activating outward current carried by Cs^{+}) and had the same duration, which was varied from 0.3 to 4.5 ms; each test pulse was followed by a step to -60 mV, eliciting inward tail current carried by Ba^{2+} . The records in **c** were obtained immediately after starting whole-cell recording (before significant effect of GTP- γ S). Both outward current at +110 mV and inward current at -60 mV were the same before and after the prepulse. The currents are for +110-mV steps of 0.3, 0.6, 0.9, 1.2 and 1.5 ms duration before (left series, 'pulse 1') and after (right series, 'pulse 2') the prepulse. Series resistance 2.5 Mohm, of which 95% was compensated. Ten minutes later (**d**) the effects of GTP- γ S were maximal. Now the prepulse significantly enhanced the inward tail current, while having almost no effect on the preceding outward current. **e**, Tail current size versus pulse duration for currents in **d**. The data from pulse 2 are fitted by a single exponential: $13,000 - 13,000 \times \exp(-\text{pulse duration}/0.31 \text{ ms})$ (consistent with complete activation of outward currents by 0.9 ms). **f**, Difference between tail currents of pulses 1 and 2 plotted against pulse duration. Data from **d**. Only the difference for pulse duration 0.9 ms or longer (when the channels were fully activated) was used. The data are fitted by a monoexponential: $2,800 \times \exp(-\text{pulse duration}/3.7 \text{ ms})$.

inhibition and thereby enhance or facilitate N-type channel currents^{4-6,13,18} (in frog sympathetic neurons, such prepulse enhancement is minimal without internal GTP- γ S⁵). The enhancement was much less in Na⁺ than in Ba²⁺ (Fig. 1d, e). The ratio between the peak Ba²⁺ and Na⁺ currents was 1.71 ± 0.05 before prepulse (in 'pulse 1') and increased to 2.49 ± 0.12 after prepulse (in 'pulse 2', $n=8$), very close to the ratio of 2.52 ± 0.08 ($n=17$) observed in control conditions with GTP rather than GTP- γ S in the pipette. Comparison suggests that the much smaller enhancement of Na⁺ currents by prepulses was due to less inhibition from the beginning, rather than less effective removal of inhibition by the prepulse.

Figure 2a, b shows measurements of instantaneous current-voltage curves with Ba²⁺ carrying inward current and Cs⁺ carrying outward current. Channels were activated by a short, strong pulse (so that channels are activated with minimal voltage-dependent unbinding of G protein with LHRH present). The outward tails were much less inhibited by LHRH than were the inward tails. Slight inhibition of outward tails was seen in cells where the inward tail was severely depressed (Fig. 2b), whereas outward tails were entirely unchanged in cells with moderate inhibition of inward tails (Fig. 2a). With GTP- γ S in the pipette, similar findings were obtained by comparing the currents before and after a prepulse. Immediately after breakthrough, the prepulse had no effect on either the outward current carried by Cs⁺ at +110 mV or the inward tail current carried by Ba²⁺ at -60 mV (Fig. 2c). After 10 min, when the effects of GTP- γ S

were maximal, the prepulse clearly enhanced the inward Ba²⁺ tail current but had no effect on the outward Cs⁺ current immediately preceding the tail (Fig. 2d). Thus, Cs⁺ passes equally well through modified channels (before prepulse) and unmodified channels (after prepulse), while Ba²⁺ currents through modified channels are smaller. As the duration of the step to +110 mV was increased (both before and after prepulse), the difference in Ba²⁺ tail currents decreased with a time constant ~ 4 ms (Fig. 2e, f), consistent with the time course of 'facilitation' (ref. 6) and with experiments in which transmitters had little effect on tails that follow relatively long (10–30 ms) steps to $> +100$ mV^{3,5}.

The differential inhibition of currents carried by Ba²⁺ or Cs⁺ suggests alteration of the ion permeation process. The changes in permeation should be most evident for tail currents following short activating pulses, where relief from G-protein inhibition is minimal. Therefore, we used noise analysis¹⁹ of tail currents activated by short pulses to estimate unitary current carried by 3 mM Ba²⁺ (Fig. 3a, b). LHRH or GTP- γ S decreased the unitary current in every cell tested. The ratio between the single-channel currents in the inhibited and uninhibited tails was ~ 0.75 with either LHRH or GTP- γ S (Fig. 3c). Because inhibited tails do not contain only modified channels, the unitary current estimated from the inhibited tails represents an average of the unitary currents through modified and unmodified channels, weighted by the current due to each population²⁰. Without knowing the currents contributed by each population, which is

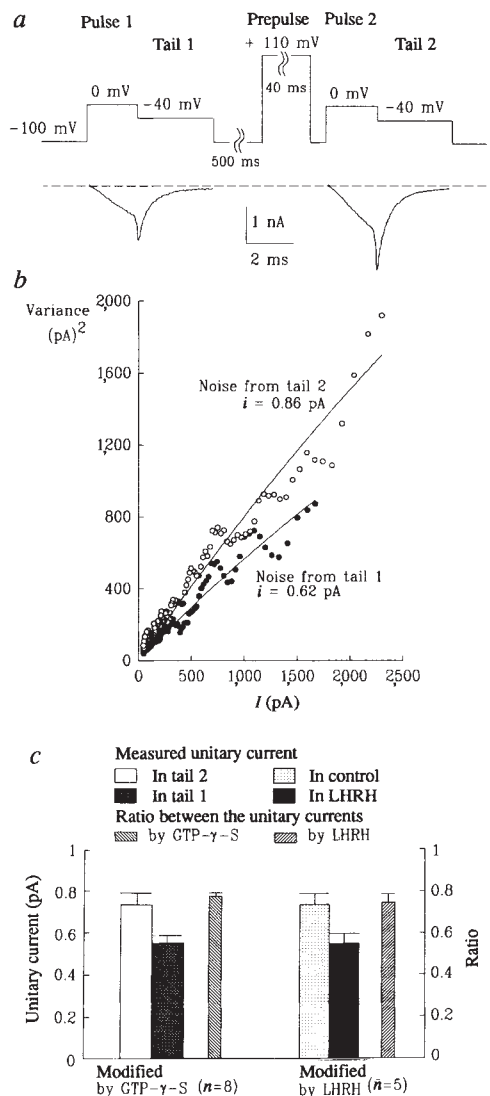


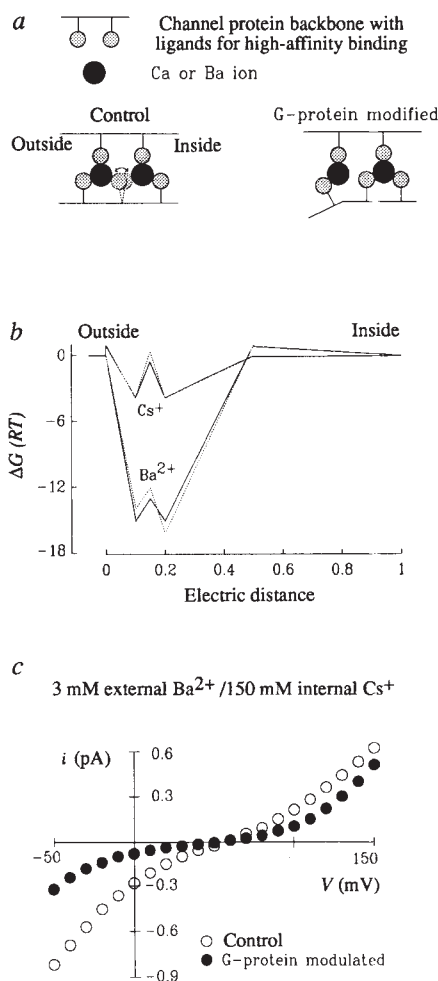
FIG. 3 Noise analysis of Ba²⁺ currents through N-type channels in the presence of GTP- γ S or LHRH. **a**, Pulse protocol and sample sweeps for noise analysis with internal GTP- γ S (0.3 mM). Channels were activated by 2-ms steps to 0 mV (pulse 1 and 2), chosen so that the voltage-dependent relief of the inhibited channels would be minimal during pulse 1 and the opening probability is small enough to ensure that the fit for the unitary currents was not significantly affected by the channel number in the cell. An upper limit on p_{open} was estimated by the ratio of the tail following the step to 0 mV to the tail following a large and long depolarization; this limit was 0.22 for the cell in **b** and was <0.35 for the other cells in **c**. Non-stationary noise analysis was done in a pairwise manner using the tail currents at -40 mV, where deactivation is relatively slow and single-channel current relatively large. Generally, ~ 100 sweeps were collected for each analysis, and care taken to ensure there was no change in the capacity transient during the series. Data were sampled at 20 μ s and filtered at 5 kHz. **b**, Variance versus mean microscopic current (I) calculated for tails 1 and 2 for 102 sweeps of a cell with internal GTP- γ S. The fitted curves were parabolas of the form: $\text{variance} = 0.62 \times I + I^2/20,000$, and $\text{variance} = 0.86 \times I + I^2/20,000$, for tails 1 and 2, respectively. **c**, Average unitary currents (wide bars, left ordinate) and ratio of modified/unmodified unitary currents (thin bars, right ordinate) with modulation by GTP- γ S or LHRH. With internal GTP- γ S, the unitary currents measured at -40 mV were 0.56 ± 0.03 pA before and 0.73 ± 0.06 pA after the prepulse; and the ratio between the two measured currents was 0.78 ± 0.02 (8 cells). Tail 1 macroscopic current was $60 \pm 4\%$ of tail 2. The product of residual series resistance and maximal current was <1.2 mV in these 8 cells. The product of residual series resistance and cell capacitance was <6 μ s. With application of LHRH (to cells with internal GTP, using the pulse protocol for pulse 1 and tail 1 only), the average unitary current at -40 mV was 0.73 ± 0.05 pA in control and 0.54 ± 0.05 pA in LHRH, and the ratio between the two currents was 0.74 ± 0.04 (5 cells). LHRH reduced macroscopic current to $51 \pm 4\%$ of control. In all 5 cells the product of residual series resistance times maximal current was <3 mV. The product of residual series resistance times cell capacitance was <7 μ s.

FIG. 4 A possible mechanism underlying the permeation change, assuming that the pore structure of N-type channels is similar to that of L channels. *a*, Two Ba^{2+} ions occupying the high-affinity Ca^{2+} binding sites compete for coordinating ligands and destabilize each other, so that the off rate from the sites with double occupancy is high enough to support substantial single-channel current²³. G protein is hypothesized to produce a conformational change at the outer high-affinity site such that the Ba^{2+} at the outer site is less well coordinated, and its competition for the common ligands between the two sites is weaker. This will allow the Ba^{2+} in the inner site to enjoy a relatively undisturbed coordination and a more stable (lower free energy) state. The off rates of the Ba^{2+} from the inner site, or the inward Ba^{2+} currents through the channel, would therefore be remarkably decreased. Monovalent ions are much less tightly bound to the sites, and their permeation through the channel does not depend on the double occupancy state or ion-ion interaction in the sites. Thus, the change in the outer site might at most represent a slightly higher energy barrier between the sites and produce no more than a small inhibition of the monovalent currents through the channel. *b*, The energy profiles across the pore for different ions based on the above rationale. The high-affinity sites are located near the outside, and the inner barrier was set at the middle of the pore (in terms of electric distance) according to the findings in L channels²⁶. The other sites and barriers in the pore are omitted for simplicity. Dotted line, G-protein-modified; solid line, control. *c*, A single channel current-voltage relation calculated from the above profiles. The universal frequency factor was set at $3 \times 10^9 \text{ s}^{-1}$ instead of $6 \times 10^{12} \text{ s}^{-1}$, considering that the nature of the reaction is aqueous diffusion²⁶. The activity coefficients of Ba^{2+} and Cs^+ are set at 0.5 and 0.75, respectively. The repulsion factors (the increase of off rates from the sites when the sites were doubly occupied as compared with the singly occupied state) are set at 10,000 for divalent-divalent, 100 for divalent-monovalent and 10 for monovalent-monovalent occupancy of the sites. Other principles were similar to those published²¹.

a complicated issue involving both gating and permeation changes, as well as the relative number of modified channels, it is impossible to calculate an exact value of unitary current for G-protein-modified channels. Therefore, 0.75 is only an upper limit of the unitary current carried by 3 mM Ba^{2+} through modified channels relative to that through unmodified channels; calculations with modelled mixtures of modified and unmodified channels show that the value can be as low as 0.25.

Figure 4 depicts a possible mechanism for how an altered permeation pathway may so differently affect the passage of different ions. Ca^{2+} channels select for Ca^{2+} or other multivalent ions by high-affinity Ca^{2+} -binding site in the pore^{21, 23} and can rapidly release the tightly bound ion in the presence of a second tightly bound ion in the sites as a result of strong ion-ion interactions which may involve ligand competition²³. A subtle change in the arrangement of the ligands could plausibly affect the permeation of tightly bound Ba^{2+} dramatically, while having minimal effects on permeation of Na^+ or Cs^+ , which are loosely bound and do not require ion-ion interaction to go through the pore. According to this model, permeation of physiological concentrations of Ca^{2+} , another tightly bound ion, would be altered similarly to low Ba^{2+} ; consistent with this prediction, LHRH inhibits currents carried by 2 mM Ca^{2+} equally as well as currents carried by 2 mM Ba^{2+} (ref. 5). This is also consistent with similar effects of transmitters on peak current carried by Ca^{2+} , Ba^{2+} or Sr^{2+} in neuroblastoma cells¹⁷. In contrast, permeation by high concentrations of Ba^{2+} or Ca^{2+} would be less affected, because both binding sites would be saturated. As expected, current carried by 150 mM Ba^{2+} was less affected by internal GTP- γ S (Fig. 1*f*) than current carried by 3 mM Ba^{2+} .

So far, single-channel recordings with neurotransmitters have shown changes in N-channel gating, but changes in unitary cur-



ent have not been observed^{2,16}. It may be that permeation changes are minimal with the high Ba^{2+} concentrations used in single-channel recording. Also, even if permeation changes are significant in high Ba^{2+} concentrations, openings of modified channels might well be intermingled with normal-sized openings of unmodified channels in long sweeps of transmitter-modulated activity; the smaller (and perhaps briefer) modified channel openings might masquerade as incompletely resolved openings rather than an obvious second population.

With maximal effects of LHRH, based on the 'extra' reduction of current carried by 3 mM Ba^{2+} compared with 150 mM Na^+ (Fig. 1), or the extra reduction of the inward limb compared with the outward limb of the instantaneous current-voltage curves (Fig. 2), we estimate that at least 40–50% of the overall reduction in current can be ascribed to permeation changes. It will be interesting to see if activation of different G proteins²⁴ gives different mixtures of effects on permeation and gating, which may contribute to the variability of phenomena such as the slow phase of activation.

It will also be interesting to see whether altered permeation is involved in the regulation of other ion channels, especially channels with relatively high-affinity binding sites in the pore. These include other types of Ca^{2+} channels, cGMP-gated channels and NMDA (*N*-methyl-D-aspartate) receptor channels, where modification of Mg^{2+} block was recently reported²⁵. □

Received 1 April; accepted 28 June 1993.

1. Dunlap, K. & Fischbach, G. J. *Physiol., Lond.* **317**, 519–535 (1981).
2. Lipscombe, D., Kongsamut, S. & Tsien, R. W. *Nature* **340**, 639–642 (1989).
3. Bean, B. P. *Nature* **340**, 153–156 (1989).
4. Pollo, A., Lovallo, M., Sher, E. & Carbone, E. *Pflügers Arch.* **422**, 75–83 (1992).
5. Boland, L. & Bean, B. P. *J. Neurosci.* **13**, 516–533 (1993).
6. Elmslie, K. S., Zhou, W. & Jones, S. W. *Neuron* **5**, 75–80 (1990).

7. Bley, K. R. & Tsien, R. W. *Neuron* **2**, 379–391 (1990).
8. Dolphin, A. C. & Scott, R. H. *Br. J. Pharm.* **88**, 213–220 (1986).
9. Scholz, K. P. & Miller, R. J. *J. Physiol., Lond.* **444**, 669–686 (1991).
10. Lester, R. A. & Jahr, C. E. *Neuron* **4**, 741–749 (1990).
11. Swartz, K. J. & Bean, B. P. *J. Neurosci.* **12**, 4358–4371 (1992).
12. Holz, G. G., Rane, S. G. & Dunlap, K. *Nature* **319**, 670–672 (1986).
13. Grassi, F. & Lux, H. D. *Neurosci. Lett.* **105**, 113–119 (1989).
14. Hescheler, J., Rosenthal, W., Trautwein, W. & Schultz, G. *Nature* **325**, 445–447 (1987).
15. Kasai, H. *J. Physiol., Lond.* **448**, 189–209 (1992).
16. Delcourt, A. H. & Tsien, R. W. *Science* **259**, 980–984 (1993).
17. Jones, S., Robins, J. & Brown, D. A. *Neurosci. Lett.* **145**, 153–156 (1992).
18. Ikeda, S. R. *J. Physiol., Lond.* **439**, 181–214 (1991).
19. Sigworth, F. J. *J. Physiol., Lond.* **307**, 97–129 (1980).
20. Sigworth, F. J. & Spalding, B. C. *Nature* **283**, 293–295 (1980).
21. Almers, W. & McCleskey, E. W. *J. Physiol., Lond.* **353**, 585–608 (1984).
22. Hess, P. & Tsien, R. W. *Nature* **309**, 453–456 (1984).
23. Kuo, C. C. & Hess, P. *J. Physiol., Lond.* **466**, 657–682 (1993).
24. Shapiro, M. S. & Hille, B. *Neuron* **10**, 11–20 (1993).
25. Chen, L. & Huang, L.-Y. *Nature* **356**, 521–523 (1992).
26. Kuo, C. C. & Hess, P. *J. Physiol., Lond.* **466**, 683–706 (1993).

ACKNOWLEDGEMENTS. We thank K. Swartz for helpful discussions. This work was supported by the NIH and the American Heart Association.

MHC-linked LMP gene products specifically alter peptidase activities of the proteasome

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PROTEASOMES are highly conserved macromolecular structures which function as endopeptidases^{1–3}. They are found in the cytoplasm and nucleus of eukaryotic tissues and consist of at least 14 non-identical subunits with molecular masses ranging from ~20 to 32K. Proteasomes are essential in the selective degradation of ubiquitinated and certain non-ubiquitinated proteins, acting as the proteolytic core of an energy-dependent 26S (1,500K) proteolytic complex. Two proteasome subunits, LMP2 and LMP7 (refs 4–7), are encoded within the major histocompatibility complex (MHC), implicating proteasomes in antigen processing^{8,9}. Here we determine the function of these two MHC-linked subunits by comparing the proteolytic activities of purified proteasomes containing (LMP⁺) or lacking (LMP[−]) these components. We find that proteasomes of both types have endopeptidase activity against substrates bearing hydrophobic, basic or acidic residues immediately preceding the cleavage site (the P1 position) and at sites following asparagine, glycine and proline residues. The activity of LMP⁺ proteasomes is much higher than that of LMP[−] proteasomes against substrates with hydrophobic, basic or asparagine residues at P1, whereas their activities are comparable when acidic and glycine residues are present at P1. The MHC-linked LMP2 and LMP7 subunits therefore function to amplify specific endopeptidase activities of the proteasome.

To determine the effect of the MHC-linked LMP2 and LMP7 subunits on proteasome activity, we compared the relative activity *in vitro* of proteasomes purified from cells expressing these two gene products with equivalent preparations from cells that do not. Proteasomes have been shown to have at least three endopeptidase activities, cleaving peptide bonds after basic, acidic and hydrophobic residues in both synthetic and natural peptides, but the effects of peptide length, the fluorogenic leaving group and the amino-end blocking group (carbamylated, suc-

cinylation or protonation) of the substrate should be taken into account. We therefore used a panel of peptide substrates that differed only in the residue immediately preceding their cleavage sites (the P1 residue).

Using proteasomes isolated from the human lymphoblastoid cell line (LCL) 721.45, which expresses LMP2 and LMP7¹⁰, we first generated a profile ranking the relative cleavage specificity for each amino acid at the P1 position. The relative hydrolysis of the peptide substrate cbz-GGL-MCA was twice that of the peptide cbz-GGF-MCA and more than five times that of cbz-GGR-MCA (Fig. 1a). Using two further sets of peptides differing only at the P1 residue, we were able to compare directly the cleavage specificity for all amino-acid residues (excluding cysteine) at this position (Fig. 1a–c). Substrates with hydrophobic P1 residues were cleaved most readily (leucine > phenylalanine > tyrosine). Peptides having basic (arginine > lysine > histidine) or acidic (aspartic acid > glutamic acid) P1 residues were cleaved less well. Surprisingly, peptides with asparagine at P1 were cleaved more readily than even the most preferred hydrophobic substrate leucine. There was also cleavage after proline and glycine.

The mutant LCL 721.174 is derived from LCL 721.45, but has a homozygous deletion of the MHC class II region, including both the LMP2 and LMP7 genes^{11,12}. Proteasomes were isolated from .174 cells and their ability to cleave the panel of synthetic peptide substrates differing at P1 was compared to that of wild-type .45 proteasomes (Fig. 2). Both .45 (LMP⁺) and .174 (LMP[−]) proteasomes displayed endopeptidase activity against peptides bearing hydrophobic, basic or acidic residues at P1, as well as activity that hydrolysed after asparagine, proline and glycine residues. LMP[−] proteasomes showed markedly (2–3-fold) less activity against hydrophobic and basic substrates, although activity against acidic, proline- and glycine-bearing substrates was nearly equivalent. Kinetic analysis indicated that the mutant .174 proteasome had the same affinity for basic or hydrophobic substrates but that the maximal velocity was decreased (data not shown). It therefore seems that incorporation of LMP subunits into the proteasome can accelerate the hydrolysis of specific peptides.

The expression of proteasome complexes containing LMP subunits is variable in normal cells and is regulated by γ -interferon (IFN- γ)^{13,14}. The hepatoma cell line H6 fails to express the LMP2 and LMP7 genes unless induced with IFN- γ (ref. 13). Proteasomes from IFN- γ -induced H6 are therefore similar to those found in .45 cells which constitutively express the LMP subunits. Conversely, proteasomes from H6 cells grown in the absence of IFN- γ should be similar to .174-derived proteasomes. Proteasomes isolated from H6 cells grown in the absence of IFN- γ gave basic and hydrophobic cleaving activities 2–3-fold lower than (LMP⁺) proteasomes from IFN- γ -induced H6 cells, but maintained wild-type activity for substrates with acidic residues at P1 (Fig. 3). Thus the absence of the two LMP subunits, either by mutation (as in .174 cells) or by normal cellular regulation (as in H6 cells), leads to differences in relative activity of proteasomes against different substrates.

Ranking the relative cleavage specificity of the proteasome on the sole basis of identity of the C-terminal residue of the peptide substrate has revealed its capacity to cleave at residues not usually considered to be good substrates (asparagine, proline and glycine). *In vivo* other substrate residues not directly flanking the scissile bond may participate in determining cleavage specificity, but our results show how regulation of the expression of individual proteasome subunits can lead to structurally distinct proteasome complexes with different endopeptidase activities. Incorporation of LMP subunits should not only generate proteasomes that cleave more readily after hydrophobic and basic residues, but should also result in the biased production of peptides bearing such residues at the C terminus. The ability of class I molecules to bind particular peptides is often dependent on the C-terminal ('anchor') residue of the peptide; so far, all class I

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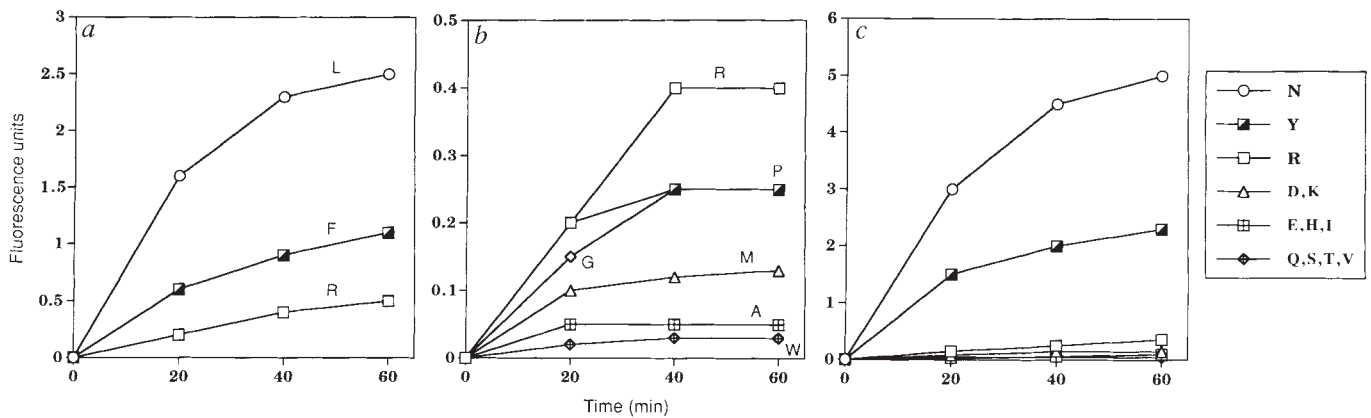


FIG. 1 Proteasome cleavage specificity is affected by the identity of the C-terminal residue of the peptide substrate. Proteasomes were isolated from 721.45 cells and activity, detected as release of the fluorescent tag, was determined using three sets of peptide substrates that differed only in the residue preceding peptide bond cleavage. Each set of peptide substrates included arginine, which was used to normalize activities between sets in Fig. 2. The individual peptides used were: a, cbz-glycine-glycine-leucine-MCA (L), cbz-glycine-glycine-phenylalanine-MCA (F) and cbz-glycine-glycine-arginine-MCA (R); b, H-arginine- β NA (R), H-proline- β NA (P), H-glycine- β NA (G), H-methionine- β NA (M), H-alanine- β NA (A), H-tryptophan- β NA (W); c, H-aspartic acid-MCA (D), H-glutamic acid-MCA (E), H-asparagine-MCA (N), H-glutamine-MCA (Q), H-valine-MCA (V), H-threonine-MCA (T), H-serine-MCA (S), H-tyrosine-MCA (Y), H-isoleucine-MCA (I), H-lysine-MCA (K), H-histidine-MCA (H) and H-arginine-MCA (R), cbz, Carbobenzoxy; MCA, 7-amido-4-methylcoumarin; β NA, β -naphthylamide.

METHODS. Cells grown to a density of $5 \times 10^7 \text{ ml}^{-1}$ were treated with 2-deoxyglucose (20 mM) and dinitrophenol (0.2 mM) for 30 min to deplete intracellular ATP pools and dissociate the 26S protease complex into its individual components²¹. Cells were pelleted by centrifugation at 5,000g for 10 min, washed twice with cold PBS and resuspended in cold PBS containing 15% sucrose, 20 mM Tris-HCl (pH 7.6), 1 mM

EDTA and 150 mM NaCl. Cells were broken using 40 strokes of a Dounce homogenizer and the resulting suspension centrifuged at 10,000g for 15 min. The supernatant was collected and centrifuged at 100,000g for 60 min. The 100,000g supernatant was then dialysed extensively against buffer A (20 mM Tris-HCl, pH 7.6, 1 mM DTT and 10% glycerol). The dialysate was applied to a Mono-Q (HR 5/5) column equilibrated with buffer A. Bound protein was eluted with a 60-ml linear gradient to 400 mM NaCl. Column fractions were assayed for ability to cleave the proteasome substrate Suc-LLVY-MCA. Active fractions were pooled, concentrated to 0.5 ml, and applied to a Superose-6 gel filtration column attached to a Pharmacia FPLC system. Chromatography was at 0.1 ml min^{-1} and fractions of 0.5 ml were collected. Upon gel filtration, the proteasome from .174 cells also eluted at 650K and showed a polypeptide profile similar to immunoprecipitates from .45 cells, with the exception of lacking LMP2 and LMP7. Assays were done at 37°C for the times indicated using $0.1 \mu\text{g}$ ($10 \mu\text{l}$) proteasome isolated from Superose-6 gel filtration. Peptide substrates were dissolved in 100% dimethyl sulfoxide and added to a final concentration of 0.5 mM. Reactions were quenched using cold ethanol, and fluorescence determined using an excitation of 380 nm and emission of 440 nm for MCA, and an excitation of 340 nm and emission of 425 nm for β NA.

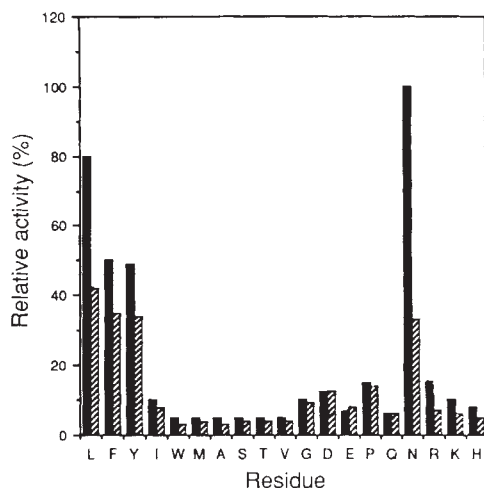


FIG. 2 Comparison of wild-type (.45; filled bars) and mutant (.174; hatched bars) proteasomes relative cleavage specificities. Proteasomes were isolated from .174 cells as described for Fig. 1. Assays were incubated for 60 min, quenched, and the fluorescence determined as for Fig. 1. Relative activity represents the ratio between the maximal activity determined for a given substrate divided by the maximal value determined for asparagine with .45 proteasomes. Activities were normalized for proteasome concentration. Similar results were obtained with two independent proteasome preparations from both cell types.

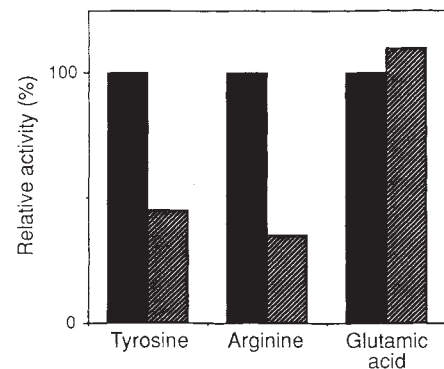


FIG. 3 Effect of IFN- γ induction on proteasome activity from the hepatoma cell line H6. Filled bars, induced activity; hatched bars, basal activity.

METHODS. H6 cells were grown in RPMI-1640 containing 10% FCS, 2 mM glutamine and 100 U ml^{-1} penicillin and 100 $\mu\text{g ml}^{-1}$ streptomycin. For induction of LMP2 and LMP7, cells were incubated in this medium with 20 U ml^{-1} IFN- γ (Genzyme) for ≥ 14 days. Proteasomes were isolated from 90 mg of cellular extract by dialysis against buffer A and separation by Mono-Q anion-exchange and Superose-6 gel filtration. Assays were done using the hydrophobic substrate succinyl-leucine-leucine-valine-tyrosine-MCA, the basic substrate cbz-alanine-arginine-arginine- β NA, or the acidic substrate cbz-leucine-leucine-glutamic acid- β NA. Relative activities are the ratios of maximal activity measured compared to proteasomes from H6 cells grown in the presence of IFN- γ . Similar results were obtained with two independent proteasome preparations, and with proteasome immunoprecipitates from both treated and untreated H6 cells.

C-terminal anchor residues have been found to be either basic or hydrophobic¹⁵⁻¹⁹.

We have demonstrated that not all proteasomes in wild-type cells contain MHC-linked LMP2 and LMP7 subunits¹³, and that IFN- γ induction can result in a higher ratio of LMP⁺ to LMP⁻ proteasomes. Together with the present results, this suggests that more class I-binding peptides could be generated in IFN- γ -treated cells. Furthermore, as not all proteasomes in normal or IFN- γ -treated cells contain LMP subunits, such cells might also produce a wider variety of peptides from a given substrate than cells that do not express LMP subunits. Hence in IFN- γ -treated cells, a larger pool of peptides might be available for binding to MHC class I molecules. The expression of other non-MHC-linked proteasome subunits might also be regulated, resulting in similar effects on function.

Two main theories concerning the function of the two MHC-linked subunits have been proposed. They might 'dock' a subset of proteasomes with the transporter associated with antigen processing (TAP) at the endoplasmic reticulum membrane, allowing direct delivery of peptides for transport into the endoplasmic reticulum lumen. These two gene products are not essential for antigen processing in general^{10,20}, indicating either that these two subunits do not perform a docking function, or that if docking occurs, it can be mediated by other proteasome subunits. Alternatively, LMP2 and LMP7 may modify the proteolytic

capabilities of the proteasome in some way that is beneficial to the immune system. Our results are more consistent with the latter hypothesis. □

Received 15 March; accepted 15 June 1993.

1. Goldberg, A. L. *Eur. J. Biochem.* **203**, 9-23 (1992).
2. Oriowski, M. *Biochemistry* **29**, 10289-10297 (1990).
3. Rivett, A. J. *Archs Biochem. Biophys.* **268**, 1-8 (1989).
4. Brown, M. G., Driscoll, J. & Monaco, J. J. *Nature* **353**, 355-357 (1991).
5. Ortiz-Navarrete, V. et al. *Nature* **353**, 662-664 (1991).
6. Glynne, R. et al. *Nature* **353**, 357-360 (1991).
7. Martinez, C. K. & Monaco, J. J. *Nature* **353**, 664-667 (1991).
8. Monaco, J. J. *Immun. Today* **13**, 173-179 (1992).
9. Michalek, M. T., Grant, E. P., Gramm, C., Goldberg, A. L. & Rock, K. L. *Nature* **363**, 552-554 (1993).
10. Arnold, D. et al. *Nature* **360**, 171-174 (1992).
11. DeMars, R. et al. *Proc. natn. Acad. Sci. U.S.A.* **82**, 8183-8187 (1985).
12. Spies, T. et al. *Nature* **348**, 744-747 (1990).
13. Brown, M. G., Driscoll, J. & Monaco, J. J. *Immun.* **151**, 1193-1204 (1993).
14. Yang, Y., Waters, J. B., Früh, K. & Peterson, P. A. *Proc. natn. Acad. Sci. U.S.A.* **89**, 4928-4932 (1992).
15. Rötzschke, O. & Falk, K. *Immun. Today* **12**, 447-455 (1991).
16. Jardeztzky, T. S., Lane, W. S., Robinson, R. A., Madden, D. R. & Wiley, D. C. *Nature* **363**, 326-329 (1991).
17. Hunt, D. F. et al. *Science* **255**, 1261-1263 (1992).
18. DiBrino, M. et al. *Proc. natn. Acad. Sci. U.S.A.* **90**, 1508-1512 (1993).
19. Sutton, J. et al. *Eur. J. Immun.* **23**, 447-453 (1993).
20. Momburg, F. et al. *Nature* **360**, 174-177 (1992).
21. Orino, E. et al. *FEBS Lett.* **284**, 206-210 (1991).

ACKNOWLEDGEMENTS. We thank R. DeMars for the 721.45 and 721.174 cell lines. This work was supported in part by grants from NIAID to J.J.M. and D.F.

γ -Interferon and expression of MHC genes regulate peptide hydrolysis by proteasomes

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THE presentation of intracellular proteins to the immune system requires their degradation to small peptides^{1,2} that then become associated with major histocompatibility complex (MHC) class I molecules^{3,4}. The generation of these peptides may involve the 20S or 26S proteasome particles, which contain multiple proteolytic activities⁵⁻¹⁴ including distinct sites that preferentially cleave small peptides on the carboxyl side of hydrophobic, basic or acidic residues^{6,13,14}. Degradation of most cell proteins requires their conjugation to ubiquitin before hydrolysis by the 26S proteasome^{6,13-16}. This large complex contains the 20S proteasome as its proteolytic core^{6,14,16-18}. This ubiquitin-dependent proteolytic pathway is implicated in MHC class I presentation^{11,12}. γ -Interferon (γ -IFN), a stimulator of antigen presentation¹, induces a subclass of proteasomes that contain two MHC-encoded subunits, LMP2 and 7 (refs 5-10). Here we show that γ -interferon alters the peptidase activities of the 20S and 26S proteasomes without affecting the rates of breakdown of proteins or of ubiquitinated proteins. By enhancing the expression of MHC genes, γ -IFN increases the proteasomes' capacity to cleave small peptides after hydrophobic and basic residues but reduces cleavage after acidic residues. Moreover, proteasomes of mutants lacking LMP subunits show decreased rates of cleavage after hydrophobic and basic residues. Thus, γ -IFN and expression of these MHC genes should favour the production by proteasomes of the types of peptides found on MHC class I molecules, which terminate almost exclusively with hydrophobic or basic residues¹⁹.

After treatment of U937 monocytic cells with γ -IFN for 3

days, the cell's proteasome content did not change, but the isolated particles hydrolysed the hydrophobic peptide Suc-LLVY-MCA two- to threefold faster, and the basic peptide Boc-LRR-MCA three- to fourfold faster than similar preparations from control cells (Table 1). γ -IFN-treatment increased the maximal rate ($V_{\max}$) at which the proteasomes could hydrolyse the hydrophobic substrate at least twofold, and the basic substrate sixfold (Fig. 1). Degradation was also faster by proteasomes from γ -IFN-treated cells of other hydrophobic peptides (0.1 mM), including Suc-LY-MCA (by 133%) and Suc-AAF-MCA (by 100%), and of other basic peptides, including Boc-GGR-MCA (by 53%) and Cbz-GKR-MCA (by 114%). In contrast, the maximal rate of cleavage by the proteasomes of the acidic substrate Cbz-LLE-MNA consistently decreased (by ~30%) after γ -IFN treatment (Fig. 1).

To investigate whether γ -IFN alters the activities of 20S and 26S proteasomes, these structures were purified by fast performance liquid chromatography (FPLC) (Table 1). Electrophoresis on non-denaturing polyacrylamide gels showed that the resulting particles were >95% pure. Accordingly, the 26S proteasome gave ATP-dependent degradation of ubiquitinated ¹²⁵I-labelled lactalbumin (but did not degrade free ¹²⁵I-labelled lactalbumin), whereas the 20S proteasome hydrolysed only the unconjugated protein. Both 20S and 26S particles from γ -IFN-treated cells cleaved the hydrophobic and basic substrates more rapidly, and the acidic substrate more slowly, than the corresponding particles from control cells (Table 1). These changes were due to alterations in the $V_{\max}$ of the purified particles, as found with the unfractionated particles (Fig. 1).

Because γ -IFN stimulates the expression of the MHC-encoded proteasome subunits LMP2 and 7, and their incorporation into proteasomes^{5,10,20}, we compared the activities of proteasomes from human B-lymphoblastoid cells and a variant that carries a homozygous MHC deletion, including the genes encoding the LMPs²¹. Although proteasome content was comparable in the two cells, the particles from the mutant degraded various hydrophobic and basic substrates 40-80% more slowly than proteasomes from wild-type cells, owing to a reduction in their $V_{\max}$ (Fig. 2; Table 2). In contrast, the acidic substrate Cbz-LLE-MNA was cleaved ~50% faster by proteasomes from the MHC-deleted variant, and the $V_{\max}$ of this process increased (Fig. 2).

TABLE 1 Effect of γ -IFN on proteolytic activities of proteasomes from U937 cells

Substrate	Total 20S and 26S			Purified 20S			Purified 26S		
	Control V	+ γ -IFN V	(% change)	Control V	+ γ -IFN V	(% change)	Control V	+ γ -IFN V	(% change)
Hydrophobic Suc-LLVY-MCA	230 $\pm$ 30	820 $\pm$ 120	(+257)*	420 $\pm$ 40	870 $\pm$ 20	(+107)*	1,870 $\pm$ 40	2,630 $\pm$ 220	(+41)*
Basic Boc-LRR-MCA	57 $\pm$ 13	250 $\pm$ 30	(+339)*	160 $\pm$ 12	430 $\pm$ 40	(+169)*	560 $\pm$ 60	1,070 $\pm$ 90	(+91)*
Acidic Cbz-LLE-MNA	125 $\pm$ 6	139 $\pm$ 40	(+11)NS	260 $\pm$ 35	226 $\pm$ 17	(-13)**	810 $\pm$ 80	440 $\pm$ 60	(-46)*
¹²⁵ I-lactalbumin	100	101 $\pm$ 10	(+1)NS	100	γ -IFN/control (%) 103 $\pm$ 17		(+3)NS	Not degraded	
Ub- ¹²⁵ I-lactalbumin	100	99 $\pm$ 5	(-1)NS	Not degraded			100	110 $\pm$ 10	(+10)NS

Proteasome fractions were prepared, and peptide substrate (0.1 mM) hydrolysis assayed (activity V expressed as nmol substrate cleaved per mg protein per hour) as for Fig. 1 (mean $\pm$ s.d.; 6 independent purifications). Similar trends were seen in crude extracts and over 96% of the soluble activity against these substrates was recovered in the total proteasome fraction (see also Table 2). 20S and 26S proteasomes (3 independent purifications) were isolated from the proteasome fractions by FPLC anion-exchange and gel-filtration chromatography²⁸. γ -IFN treatment for 3 d increased the LMP2 $\sim$ 30-fold and LMP7 2-fold in total proteasome particles and in purified 20S and 26S particles. We measured the LMP2 and 7 proteins by western blotting in at least 3 preparations using specific antibodies (gift from K. Tanaka). Degradation of ubiquitinated lactalbumin was assayed¹⁶ in the presence of 2 mM ATP and free ¹²⁵I-lactalbumin in the presence of apyrase (5 U ml⁻¹). Five to ten per cent of the ¹²⁵I-labelled protein was digested to acid-soluble fragments. To normalize these data between preparations, the activity from control cells was taken as 100%. * P <0.01; ** P <0.05; NS not significant (t-test).

Suc-LLVY-MCA, succinyl-Leu-Leu-Val-Tyr-7-amido-4-methylcoumarin; Boc-LRR-MCA, N-tert-butoxycarbonyl-Leu-Arg-Arg-7-amido-4-methylcoumarin; Cbz-LLE-MNA, benzylcarboxyl-Leu-Leu-Glu-p-nitroanilide.

Thus, the expression of MHC-encoded genes in the lymphoblasts and γ -IFN treatment of U937 cells affected the peptidase activities of proteasomes similarly. These observations suggest that γ -IFN alters proteasomal peptidase activities by stimulating the expression of MHC-encoded subunits. Accordingly, in wild-type lymphoblasts, as in U937 monocytes, γ -IFN treatment increased the degradation of hydrophobic and basic substrates, but in the MHC-deletion mutant, γ -IFN did not alter the degradation of any basic substrate tested, and caused only a small increase in the cleavage of various hydrophobic peptides (13–35% of that seen in the wild type) (Table 2). Surprisingly, in the mutant γ -IFN stimulated hydrolysis of the acidic substrate, even though in the wild-type lymphoblasts and U937 cells, γ -IFN reduced Cbz-LLE-MNA breakdown (Tables 1 and 2).

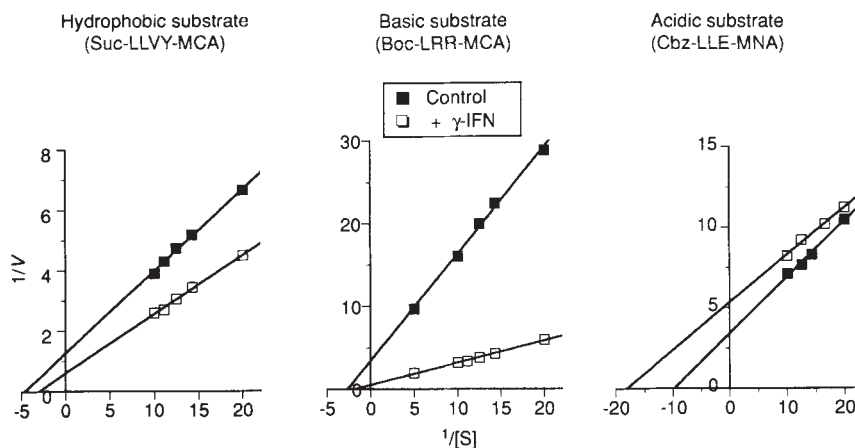
The 20S proteasome has generally been viewed as a single structure with a uniform set of activities. These experiments, however, demonstrate their functional heterogeneity and also that their various peptidase activities can be differentially regulated. *In vivo* these activities probably change in different physiological conditions, for example during infections when γ -IFN stimulates immune responses^{22,23}. Presumably, γ -IFN affects each peptidase activity differently, because it alters the subunit composition of the particle²⁰. These alterations in peptidase activities require the MHC region containing the LMP genes whose expression is stimulated by γ -IFN^{5,10,20}. Also, deletion of

this region causes functional changes opposite to those induced by γ -IFN. Furthermore, western blot analysis shows that γ -IFN treatment increases the amount of LMP2 and 7 in proteasomes of 721 cells and in both the 20S and 26S particles from U937 cells (Tables 1 and 2). It therefore appears most likely that incorporation of these LMP subunits causes the functional alterations.

Incorporation of the MHC-encoded subunits may enhance the activities of pre-existent tryptic- and chymotryptic-like sites, or these subunits may themselves contain these catalytic activities. Interestingly, a yeast proteasome subunit necessary for the chymotryptic-like activity shares considerable homology with LMP7 (ref. 24). Presumably the incorporation of the LMPs replaces the peptidyl glutamyl subunits or inhibits their activity. But in the MHC-deletion mutant, γ -IFN causes a large increase in this activity and a small increase in chymotryptic activity. These responses may be explained by the ability of γ -IFN to cause additional changes in the pattern of proteasome subunits²⁰ that do not involve LMPs.

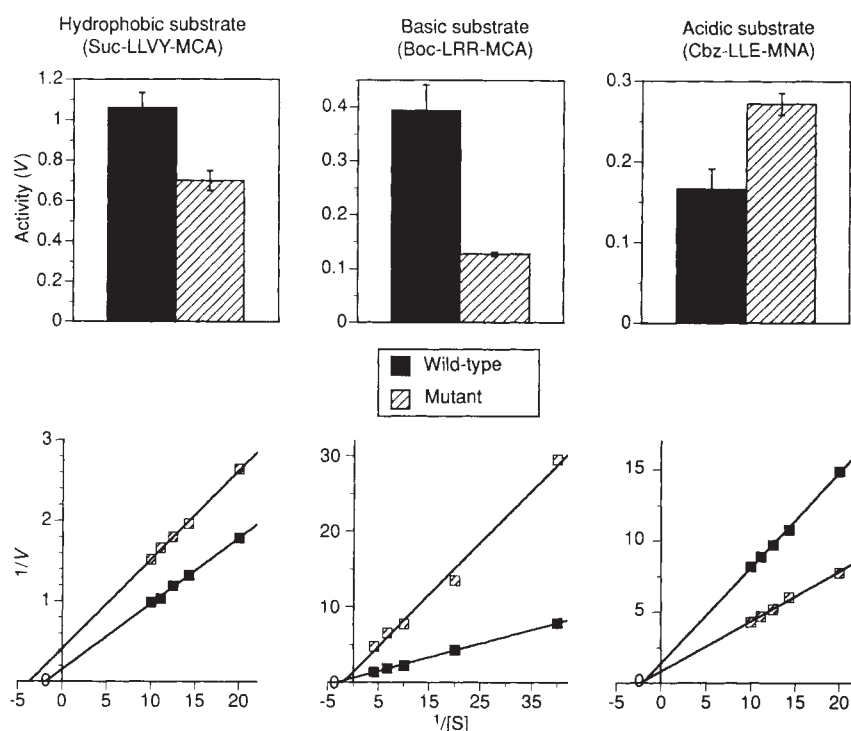
Neither γ -IFN treatment nor the MHC deletion alters the overall rates of digestion by proteasomes of lactalbumin or ubiquitin-conjugated lactalbumin to acid-soluble fragments (Table 1; Fig. 2). These findings indicate that the initial cleavages of polypeptides by the 20S proteasome or of ubiquitinated proteins by the 26S proteasome are not altered by γ -IFN or by

FIG. 1 Effect of γ -IFN on peptidase activities of proteasomes from U937 cells. Data shown are rates of hydrolysis of fluorogenic substrates¹⁸ by proteasomes prepared by differential centrifugation of homogenates of control cells and cells treated for 3 days with 1,000 U ml⁻¹ of human γ -IFN. Proteasome fractions were obtained by centrifugation of soluble extracts²⁸ for 5 h at 100,000g. After solubilizing pellets, rates of cleavage (V) by proteasome fractions were analysed with different substrate concentrations ([S], in mM) in the presence of 5 U ml⁻¹ apyrase. V_{max} values: for Suc-LLVY-MCA, 0.7 units (where one unit represents one nmol substrate cleared per mg protein per hour) and 1.6 after γ -IFN treatment; for Boc-LRR-MCA, 0.3 units and 1.9 with γ -IFN; for Cbz-LLE-MNA, 0.3 units and 0.2 with γ -IFN. K_m : for Suc-LLVY-MCA, 0.2 mM and 0.3 mM with γ -IFN; for Boc-LRR-MCA, 0.4 mM and 0.5 mM with γ -IFN; for Cbz-LLE-MNA, 0.10 mM and 0.06 mM with γ -IFN. A similar kinetic analysis was obtained for purified 20S proteasomes (Table 1): V_{max} with Suc LLVY-MAC of 1.2 and 2.5 after γ -IFN,



and with BOC LRR-MCA of 0.7 and 4.1 after γ -INF (K_m values of 0.4 nM and 0.5 nM, respectively.) Similar results were obtained in two independent experiments.

FIG. 2 Proteasomes of wild-type (721) and MHC-deficient (721.174) lymphoblastoid cells differ in their peptidase activities. Top, rates of hydrolysis of three substrates (0.1 mM) were measured in proteasome fractions, as for Fig. 1. Differences in all three activities were statistically significant ($P < 0.01$, mean $\pm$ s.d.; $n = 4$ independent purifications). Bottom, kinetic analysis for all the substrates (mM) by proteasomes from wild-type and mutant cells. $V_{\max}$ (units of activity defined in Fig. 1 legend): for Suc-LLVY-MCA, 6.5 units for the wild type versus 2.4 for the mutant; for Boc-LRR-MCA, 1.6 units versus 0.7; for Cbz-LLE-MNA, 0.7 units versus 1.2. K_m : for Suc-LLVY-MCA, 0.5 mM (wild type) versus 0.3 mM (mutant); for Boc-LRR-MCA, 0.3 mM versus 0.5 mM; for Cbz-LLE-MNA, 0.4 mM (wild type and mutant). Similar results were obtained in two independent experiments. No significant differences in the rates of digestion of ^{125}I -labelled lactalbumin or Ub- ^{125}I -lactalbumin (Table 1) were found. Mutant proteasomes degraded ^{125}I -labelled lactalbumin at $98 \pm 10\%$ of the wild-type rate and Ub- ^{125}I -lactalbumin at $101 \pm 4\%$ ($n = 4$ independent experiments).



MHC expression, which instead seems to affect the subsequent cleavages of the acid-soluble fragments to short peptides. These changes in peptidase activities should alter the products generated by proteasomes during protein degradation, and should favour the production of peptides ending with basic or hydrophobic carboxyl termini, while reducing the frequency of peptides with acidic termini. Thus γ -IFN and MHC expression can promote the generation of just those types of peptides that bind to MHC class I molecules. The carboxy-terminal residue of an antigenic peptide plays a key role in binding to specific MHC class I molecules^{19,25}. Nearly all peptides found associated with class I molecules have hydrophobic or basic carboxy termini, and virtually none has an acidic carboxy terminus¹⁹. These

observations indicate that cleavages by the proteasome may determine the nature of the carboxy termini of the peptides associated with MHC class I proteins. In contrast, residues preceding the amino terminus of a class I-associated peptide show no predominance of basic and hydrophobic residues¹⁹ and therefore these amino termini are probably generated by a distinct peptidase activity.

It has been proposed that proteasomes are not essential for MHC class I antigen presentation on the basis that lymphoblast mutants lacking LMP subunits can still present antigens^{26,27}. But the proteasomes in our MHC-deletion strain cleave peptides after basic and hydrophobic residues (Table 1; Fig. 2), and they do so even faster than proteasomes from control U937 cells. These basal, MHC-independent activities of the proteasome may explain how cells lacking LMPs can still present antigens on MHC class I. Our findings predict that the lack of LMPs should only reduce the efficiency of antigen presentation and not block this process, as has been observed²⁷. Conversely, the γ -IFN-induced, MHC-dependent changes in proteasome activities, by favouring the production of peptides appropriate for binding to MHC class I molecules should enhance the efficiency of antigen presentation. □

TABLE 2 Comparison of γ -IFN-induced changes in peptidase activities of proteasomes from wild-type (721) and MHC-deficient (721.174) cells

Substrate	Increase in activity induced by γ -IFN			
	Wild-type cells		MHC-deficient mutant	
	V	(% change)	V	(% change)
Hydrophobic				
Suc-LLVY-MCA	340	(+33)*	120	(+18)**
Suc-LY-MCA	66	(+34)*	10	(+26)**
Suc-AAF-MCA	62	(+42)*	8	(+15)**
Basic				
Boc-LRR-MCA	180	(+48)*	6	(+5)NS
Boc-GKR-MCA	1.6	(+28)*	0.2	(+6)NS
Cbz-GGR-MCA	1.2	(+34)*	0.08	(-5)NS
Acidic				
Cbz-LLE-MNA	-40	(-24)*	+80	(+35)**

Data are mean differences in activity (V, in nmol substrate cleaved per mg protein per hour) between proteasome fractions from control cells and cells treated with γ -IFN for 3 d. With all hydrophobic and basic substrates, γ -IFN caused larger changes in wild-type cells (3 independent experiments); with acidic substrate, γ -IFN consistently caused opposite changes in wild-type and mutant cells. γ -IFN increased approximately 2-fold the amounts of both LMP2 and 7 in wild-type proteasomes, as shown by western blot analysis of 4 preparations. No LMP2 or 7 was detectable in the mutants. * $P < 0.02$; ** $P < 0.05$; NS, difference not significant (t-test).

Received 16 April; accepted 19 August 1993.

1. Townsend, A. R. M. & Bodmer, H. A. *Rev. Immun.* **7**, 601-624 (1989).
2. Morrison, L. A., Lukacher, A., Braciale, V. L., Fan, D. P. & Braciale, T. J. *J. exp. Med.* **163**, 903-921 (1986).
3. Monaco, J. J. *Immun. Today* **13**, 173-179 (1992).
4. Spies, T. & DeMars, R. *Nature* **351**, 323-324 (1991).
5. Driscoll, J. & Finley, D. *Cell* **68**, 823-825 (1992).
6. Goldberg, A. L. & Rock, K. L. *Nature* **357**, 375-379 (1992).
7. Martinez, C. K. & Monaco, J. J. *Nature* **353**, 664-667 (1991).
8. Ortiz-Navarrete, V. et al. *Nature* **353**, 662-664 (1991).
9. Kelly, A. et al. *Nature* **353**, 667-668 (1991).
10. Brown, M. G., Driscoll, J. & Monaco, J. J. *Nature* **353**, 355-357 (1991).
11. Michalek, M. T., Grant, E. P., Gramm, C., Goldberg, A. L. & Rock, K. L. *Nature* **363**, 552-554 (1993).
12. Townsend, A. et al. *J. exp. Med.* **168**, 1211-1224 (1988).
13. Goldberg, A. L. *Eur. J. Biochem.* **203**, 9-23 (1992).
14. Rivett, A. J. *Archs Biochem. Biophys.* **218**, 1-8 (1989).
15. Finley, D. & Chau, V. A. *Rev. Cell Biol.* **7**, 25-69 (1991).
16. Waxman, L., Fagan, J. M. & Goldberg, A. L. *J. biol. Chem.* **262**, 2451-2457 (1987).
17. Armon, T., Ganoth, D. & Herskko, A. *J. biol. Chem.* **265**, 20723-20726 (1990).
18. Driscoll, J. & Goldberg, A. L. *J. biol. Chem.* **265**, 4789-4792 (1990).
19. Falk, K. & Rotzschke, O. *Sem. Immun.* **5**, 81-94 (1993).
20. Yang, Y., Waters, J. B., Fruh, R. & Peterson, P. A. *Proc. natn. Acad. Sci. U.S.A.* **89**, 4928-4932 (1992).

21. DeMars, R. et al. *Proc. natn. Acad. Sci. U.S.A.* **82**, 8183–8187 (1985).
22. Ahn, J. Y. et al. *J. biol. Chem.* **266**, 15746–15749 (1991).
23. Scherrer, K. *Molec. Biol. Rep.* **14**, 1–9 (1990).
24. Heinemeyer, W., Kleinschmidt, J. A., Saidowsky, J., Escher, C. H. & Wolf, D. *EMBO J.* **10**, 555–562 (1991).
25. Parham, P. *Nature* **360**, 300–301 (1992).
26. Arnold, D. et al. *Nature* **360**, 171–174 (1992).
27. Momburg, F. et al. *Nature* **360**, 174–177 (1992).
28. Tanaka, K., Li, K., Ichihara, A., Waxman, L. & Goldberg, A. L. *J. biol. Chem.* **261**, 15197–15203 (1986).

ACKNOWLEDGEMENTS. We thank R. DeMars for lymphoblastoid lines, K. Tanaka for antibodies, and A. Scott for secretarial assistance.

A role for sialyl Lewis-X/A glycoconjugates in capillary morphogenesis

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To identify cell adhesion molecules required for angiogenesis, we used an *in vitro* model in which bovine capillary endothelial cells can be induced to form capillary-like tubes. Monoclonal antibodies directed against the carbohydrate epitopes sialyl Lewis-X and sialyl Lewis-A inhibited capillary formation. We postulated that a member of the selectin family of adhesion molecules may be involved in capillary formation because these proteins bind to sialyl Lewis-X/A-containing ligands. We isolated a 2.8-kilobase complementary DNA from a bovine capillary endothelial cell cDNA library which encodes a polypeptide with 71% identity to human E-selectin. We report here that antibody directed against the bovine E-selectin inhibited capillary formation, suggesting that in addition to its role in leukocyte adhesion to endothelium, a form of E-selectin is involved in capillary morphogenesis.

Angiogenesis occurs during development and in adult life during wound healing and endometrial proliferation. Abnormal capillary growth underlies many disease states and represents a stimulation of endothelial cells to migrate into the perivascular space and form new capillaries¹. As cell-surface oligosaccharides can mediate interactions between mammalian cells, we examined their role in capillary tube formation. One strategy was to test the effect of nine different anti-carbohydrate IgMs on tube formation. The epitopes, present on glycolipids and glycoproteins, consist of three to six monosaccharides, and most are named after the blood group antigens they represent². Antibodies directed against sialyl Lewis-X (NeuAca2,3Galβ1,4(Fuca1,3)-IcNAc), and sialyl Lewis-A (NeuAca2,3Galβ1,3(Fuca1,4)-GlcNAc) inhibited tube formation (Fig. 1c and d, respectively) compared to untreated cells (Fig. 1a) in that the branching network was not observed. Antibodies directed against Lewis-X, Lewis-B, Lewis-A, A, B, H, and Vim2, however, had no effect (Fig. 1b shows an example). For each antibody, binding sites were quantified, and with the exception of anti-Vim2, all bound to bovine capillary endothelial (BCE) cells induced to form tubes or in monolayers (Fig. 1e). Anti-Lewis-B and anti-Lewis-A exhibited the highest binding to BCE cells but did not affect tube formation. This result indicates that number of epitopes does not correlate with ability to block tube formation and suggests that anti-sialyl Lewis-X/A antibodies disrupt specific interactions.

Two cell adhesion molecules, E-selectin and P-selectin, bind

to sialyl Lewis-X-containing ligands on neutrophils via lectin domains present at their amino termini^{3–6}. This interaction initiates binding of these cells to activated endothelium^{7,8}. *In vitro*, E-selectin also binds to sialyl Lewis-A^{9–11}. As the results in Fig. 1 suggested that a selectin may be involved in capillary formation, we analysed BCE cells for E-selectin-related RNAs using a human E-selectin cDNA (Fig. 2a). RNA from activated human umbilical vein endothelial cells (HUVEC) served as a positive control for detection of E-selectin messenger RNA (Fig. 2, lane

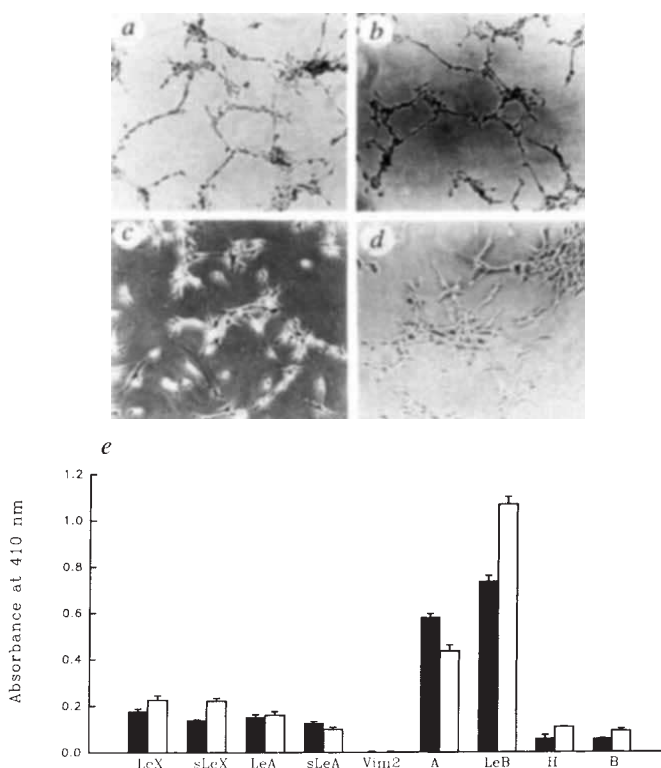


FIG. 1 Effect of anti-carbohydrate IgMs on capillary tube formation. a, BCE cells induced to form tubes; b, BCE cells induced to form tubes in presence of anti-Lewis-B; c, anti-sialyl Lewis-X; and d, anti-sialyl Lewis-A. To induce tube formation, serum-starved BCE cells were plated on 1-cm² fibronectin-coated bacteriological wells in serum-free tube media^{19,21}. Antibodies were added 20 h after plating in 100 µl fresh media. Cells were fixed with 0.25% glutaraldehyde 44 h after plating. Phase-contrast images were obtained using a Diaphot Nikon inverted microscope. A 1/500 dilution of anti-sialyl Lewis-X ascites (CSLEX1, American Type Culture Collection) and 1/500 dilution of anti-sialyl Lewis-A ascites (UCLA Tissue Typing Laboratory) were used. Anti-Lewis-A, Lewis-B, A, H, B (Chembiomed Inc., Edmonton, Alberta) and Vim-2 (An Der Grub Bioresearch, Daumberg, Austria) were tested at 10 µg ml⁻¹. Anti-Lewis-X ascites (Developmental Studies Hybridoma Bank, Department of Pharmacology, Johns Hopkins University School of Medicine, Baltimore, MD) at 1/500 or 1/200 dilution had no effect indicating that ascites does not impair tube formation (magnification, 50×). e, Binding of anti-carbohydrate antibodies to capillary endothelial cells. An enzyme-linked immunoassay was used to determine the abundance of epitopes on BCE monolayers (black bars) and BCE tubes (white bars). The graphs represent the average of triplicate assays for monolayers and quintuplicate for tubes with the standard deviation indicated. For monolayers, BCE cells were plated on gelatin-coated dishes in growth medium^{19,21} for 32 h. For tubes, BCE cells were plated onto fibronectin-coated dishes in tube medium for 32 h. Cells were scraped from dishes in ice-cold phosphate-buffered saline (PBS), 5 mM EDTA, sedimented onto a poly-L-lysine-coated 96-well dish (7 × 10⁴ cells per well), fixed with 0.25% glutaraldehyde/PBS²², and carbohydrate epitopes were measured²³.

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1) (ref. 12). As expected, no signal was detected in unactivated HUVEC (Fig. 2, lane 2). An RNA species of ~3 kilobases (kb) was detected in all BCE RNA preparations (Fig. 2, lanes 3–8) but not in aortic endothelial RNA (Fig. 2, lane 9). The signal was increased fourfold in BCE monolayers activated with tumour necrosis factor- α (TNF- α) (lane 3) and 2–3-fold in BCE cells induced to form tubes (lanes 6 and 8).

To characterize the 3-kb bovine E-selectin-related RNA, we prepared a cDNA library¹³ from BCE cells induced to form tubes. Two distinct cDNA clones were isolated and sequenced^{14,15}. The first cDNA clone consists of 2,850 base pairs (bp) with an open reading frame of 1,455 bp. The predicted product of this cDNA has significant homology to human E-selectin (Fig. 3). The second cDNA (2,730 bp) is similar to

human P-selectin¹⁵. Thus, the 3-kb signal observed in Fig. 2a represented a composite of two RNA species. Therefore, we re-analysed RNA from BCE cells using the bovine E-selectin cDNA (Fig. 2c). The conditions used were such that the cDNA probe did not hybridize with P-selectin RNA. Bovine capillary E-selectin mRNA increased 10-fold upon capillary tube induction (lanes 3, 4) compared to control BCE cells (lanes 1, 2).

The polypeptide encoded by the 2.85-kb cDNA was aligned with human E-selectin (Fig. 3). The sequences are 71% identical if a 126-amino-acid gap is introduced into the bovine sequence. This gap corresponds to the consensus repeat (CR) domains 4 and 5 of human E-selectin. Hence, the bovine capillary form of E-selectin contains each of the domains found in the selectins, but differs from human E-selectin in that it contains only four

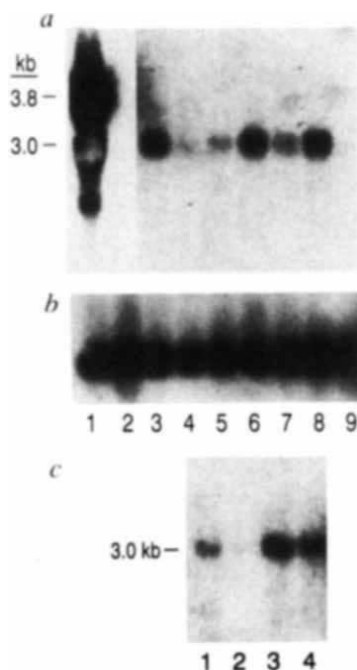


FIG. 2 Northern blot analysis of HUVEC and BCE RNAs. RNA was isolated from the following cells. HUVEC were incubated for 3 h in the presence (lane 1) or absence (lane 2) of 200 units ml^{-1} TNF- α . BCE monolayers in growth medium were incubated for 3 h in the presence (lane 3) or absence (lane 4) of TNF- α . BCE cells were plated in growth medium (lanes 5 and 7) or induced to form tubes (lanes 6 and 8). Bovine aortic endothelial cells were grown in Dulbecco's modified Eagle's medium with 10% calf serum, penicillin and streptomycin (lane 9). Poly(A)⁺-selected RNA was isolated using a proteinase K/SDS method²⁴, fractionated by formaldehyde-1% agarose gel electrophoresis, and transferred to nitrocellulose¹⁴. The northern blot (a) was incubated with a ³²P-labelled fragment (nucleotides 1–561) of the human E-selectin cDNA in 30% formamide, 50 mM NaPO₄, pH 7.4, 750 mM NaCl, 1 mM EDTA, 5 × Denhardt's¹⁴, 0.1% SDS, 100 $\mu\text{g ml}^{-1}$ poly(A)⁺, and 100 $\mu\text{g ml}^{-1}$ heat-denatured salmon sperm DNA at 42 °C and then washed in 30% formamide, 75 mM sodium citrate, pH 7, 750 mM NaCl, 0.1% SDS for 4 × 20 min at 42 °C. The blot was then probed with ³²P-labelled glyceraldehyde-3-phosphate dehydrogenase cDNA²⁵ for an internal standard (b) and the signals obtained were quantified by phosphor imager analysis²⁶. For this figure, the blot was autoradiographed. Lanes 1 and 2 in a have had a shorter exposure than lanes 3–9. In c, RNA from BCE cells plated on gelatin-coated dishes in growth medium (lane 1), on gelatin-coated dishes in tube medium (lane 2), or on fibronectin-coated dishes in tube medium (lanes 3 and 4) was analysed as in a, except that a 1.7-kb *KpnI* fragment of the bovine E-selectin cDNA was used as a probe, the hybridization solution contained 50% formamide, and washes were in 30 mM sodium citrate, pH 7, 300 mM NaCl, 0.1% SDS at 50 °C. Quantification was as described.

FIG. 3 Alignment of deduced amino-acid sequence of bovine capillary E-selectin cDNA with human E-selectin. The polypeptides were aligned with a pattern-induced multi-sequence alignment programme²⁷. Dashes represent aligned identical amino acids; dots represent gaps introduced to maintain the alignment, and amino-acid differences in human sequence are indicated. Based on homology to human E-selectin, the polypeptide domains of bovine capillary E-selectin are assigned as follows: amino acids 1–22, signal peptide; 23–142, lectin domain; 143–177, EGF-like domain; 431–453, transmembrane domain; and 454–485, cytoplasmic domain. Amino acids 178–422 can be divided into four CR domains of 61–63 amino acids, which correspond to CR domains 1, 2, 3 and 6 of human E-selectin.

Bovine	: MIVSQYLSALTFLVLLLFKESRTWSYHASTEMMTFEEARDYCQKTYTALVAIQNEIEIYL	60
Human	: --A--F-----L---I---GA---NT---A--YD--SA---QR--H-----K-----	59
Bovine	: NSTFSYSPSYWIGIRKINGTWTWIGTNKSLTKEATNWPAGPEPNKQSDCEDCVIYIKRE	120
Human	: --IL-----V-NV-V-V--Q-P--E--K-----R-K-----	119
Bovine	: KDSGKWNDEKCTKQKALCYKAACNPTPCGSHGECVETINNYTCQCHPGFKGLKCEQVVT	180
Human	: --V-M---R-S-K-----T---TN-S-SG-----K-D---S-----I-N	179
Bovine	: CPAQKHPEHGLHVC.NPLGKFTYNSSCSISCAEGYLPSSTEATRCMSSGEWSTPLPKCNV	239
Human	: -T-LES---S---SH---N-S-----DR-----M-TMQ-----A-I-A---	239
Bovine	: VKCDALSNLDNGVNVNCSFNHSGSLPWNTTCTFECEGYKLTGPQHLQCTSSGIWDNKQPTC	299
Human	: -E---VT-PA---F-E-FQ-P--F-----D-E--FE-M-A-S-----N---EK---	299
Bovine	: KAVSCAAISHPQNGTVNCSHVSVDGFAFKSSCHFTCAEGFTLQGPQTVECTAQGGWTQRV	359
Human	: ---T-R-VRQ---S-R---PA-E-T-----N---E---M---A---T-----QI	359
Bovine	: PVCE.....	363
Human	: ---afqctalsnpergymnclpsasgsfrygsscefsceggfvlkgskrlqcgptgwd	419
Bovine	:	479
Human	: nekptceavrcdvhqppkgvlrcahspigeftyksscafsceegfelhgstqlctsqg	479
Bovine	:VVRCSRLDVSGLNMNCSGEPVLGTCTFACPERWTLNGSVVLTTCGATGH	413
Human	: qwteevpscq--K--S-A-P--I--S-----F--V-K-----G-----AAR-----	539
Bovine	: WSGMLPTCEAPTVSQTPLAVGLSTAGVSLVTIPSLFWLLKRLQKKAKKFSAPSSCSSLK	473
Human	: ---L-----E-NI--VA---A--L--L-LAP--L--RKCLR-----V-----Q--E	598
Bovine	: SNGCYSTPSKLI	485
Human	: -D-S-QK--YIL	610

CR domains. *In vivo*, human E-selectin has been detected in post-capillary venules at sites of activation by immune or inflammatory events^{16,17}. *In vitro*, human E-selectin is most often studied in cytokine-stimulated HUVEC because its mRNA and protein are undetectable in unactivated HUVEC¹². In contrast, bovine capillary E-selectin mRNA is expressed constitutively in BCE cells (Fig. 2). The capillary E-selectin cDNA may represent the bovine counterpart of the human E-selectin gene and if so, our data suggest that, in addition to its role in leukocyte adhesion to activated endothelium, E-selectin functions in capillary formation and/or maintenance.

To test the function of bovine E-selectin directly, we raised an antibody against a peptide that corresponds to amino acids 2–19 of the lectin domain. This region is important for the carbohydrate-binding activity of human E-selectin¹⁸ and therefore provides a likely epitope for an antibody capable of blocking bovine E-selectin function. The antibody inhibits Ca^{2+} -dependent binding of HL-60 cells, a human leukaemia cell line with abundant sialyl Lewis-X epitopes, to BCE cells and recognizes a 130K polypeptide in BCE cells (data not shown). IgG purified from this antiserum markedly inhibited capillary tube formation (Fig. 4b) compared to untreated cells (a), preimmune IgG (c), or anti-bovine P-selectin IgG (d). BCE cells induced to form tubes in the presence of anti-E-selectin IgG exhibited an adherent, flattened morphology with some long processes, but the capillary network seen in Fig. 4a, c and d was not observed.

We propose that E-selectin participates in capillary morphogenesis by binding to a BCE cell sialyl Lewis-X and/or sialyl Lewis-A-containing ligand. Antibodies directed against these tetrasaccharides inhibit tube formation by competing with bovine E-selectin. Anti-bovine capillary E-selectin inhibits capillary formation by blocking the ability of the E-selectin to bind to its sialyl Lewis-X/A ligand. In support of this hypothesis,

we have identified asparagine-linked oligosaccharides containing sialyl Lewis-X/A-like determinants in BCE cells¹⁹. The increase in E-selectin mRNA upon tube induction suggests that its expression is part of the capillary differentiation programme. A deficiency in neutrophil sialyl Lewis-X in patients suffering from mental retardation, dwarfism, and infections²⁰ suggests that these structures are required for normal development. □

Received 10 March; accepted 25 June 1993.

1. Folkman, J. & Klagsbrun, M. *Science* **235**, 442–447 (1987).
2. Feizi, T. *Nature* **314**, 53–57 (1985).
3. Lowe, J. et al. *Cell* **63**, 475–484 (1990).
4. Phillips, M. L. et al. *Science* **250**, 1130–1132 (1990).
5. Walz, E. L., Aruffo, A., Kolanus, W., Bevilacqua, M. & Seed, B. *Science* **250**, 1132–1135 (1990).
6. Polley, M. J. et al. *Proc. natn. Acad. Sci. U.S.A.* **88**, 6224–6228 (1991).
7. McEver, R. P. *Thromb. Haemostasis* **66**, 80–87 (1991).
8. Bevilacqua, M. P. & Nelson, R. M. *J. clin. Invest.* **91**, 379–387 (1993).
9. Berg, E. L., Robinson, M. K., Mansson, O., Butcher, E. C. & Magnani, J. I. *J. biol. Chem.* **266**, 14869–14872 (1991).
10. Takada, A. et al. *Biochem. biophys. Res. Commun.* **179**, 713–719 (1991).
11. Tyrrell, D. et al. *Proc. natn. Acad. Sci. U.S.A.* **88**, 10372–10376 (1991).
12. Bevilacqua, M. P., Stengelin, S., Gimbrone, M. A. & Seed, B. *Science* **243**, 1160–1164 (1989).
13. Aruffo, A. & Seed, B. *Proc. natn. Acad. Sci. U.S.A.* **84**, 8573–8577 (1987).
14. Sambrook, J., Maniatis, T. & Fritsch, E. F. *Molecular Cloning: a Laboratory Manual* 2nd edn (Cold Spring Harbor Laboratory Press, New York, 1989).
15. Strubel, N. A., Nguyen, M., Kansas, G. S., Tedder, T. F. & Bischoff, J. *Biochem. biophys. Res. Commun.* **192**, 338–344 (1993).
16. Cotran, R., Gimbrone, M. A., Bevilacqua, M. P., Mendrick, D. L. & Pober, J. S. *J. exp. Med.* **164**, 661–666 (1986).
17. Messadi, D. V., Pober, J. S., Fiers, W., Gimbrone, M. A. & Murphy, G. F. *J. Immun.* **139**, 1557–1562 (1987).
18. Erbe, D. V. et al. *J. Cell Biol.* **119**, 215–227 (1992).
19. Nguyen, M., Folkman, J. & Bischoff, J. *J. biol. Chem.* **267**, 26157–26165 (1992).
20. Etzioni, A. et al. *New Engl. J. Med.* **327**, 1789–1791 (1992).
21. Ingber, D. & Folkman, J. *J. Cell Biol.* **109**, 317–330 (1989).
22. Heussen, C. H., Stocker, J. W. & Gisler, R. H. *Meth. Enzym.* **73**, 406–418 (1981).
23. Zhou, Q. et al. *J. Cell Biol.* **115**, 557–564 (1991).
24. Gonda, T. J., Sheiness, D. K. & Bishop, J. M. *Molec. cell. Biol.* **2**, 617–624 (1982).
25. Fort, P. et al. *Nucleic Acids Res.* **13**, 1431–1442 (1985).
26. Johnston, R. F., Pickett, S. C. & Barker, S. I. *Electrophoresis* **11**, 355–360 (1990).
27. Smith, R. F. & Smith, T. F. *Proc. natn. Acad. Sci. U.S.A.* **87**, 118–121 (1990).

ACKNOWLEDGEMENTS. We thank J. Lowe and C. Corless for anti-carbohydrate antibodies. Recombinant basic fibroblast growth factor was provided by Takeda Chemical Industries, Osaka, Japan. We also thank B. Thorens for advice on synthesis of the cDNA library. The nucleotide sequence of the bovine E-selectin has been submitted to GENBANK under accession number L12039.

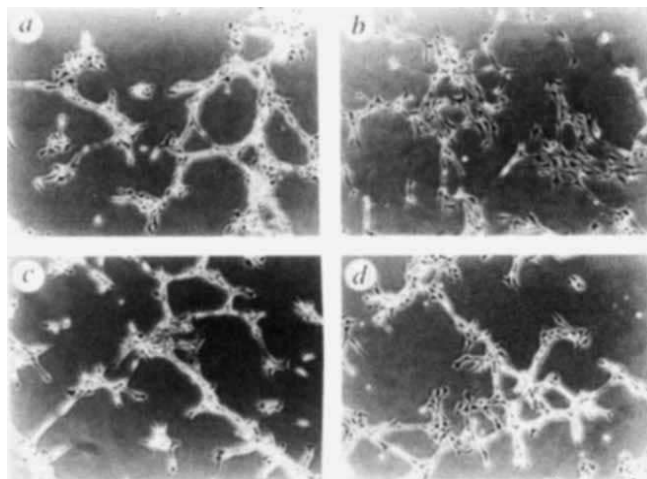


FIG. 4 Effect of anti-bovine capillary E- and P-selectin IgGs on capillary tube formation *in vitro*. a, BCE cells were induced to form tubes; b–d, BCE cells were induced to form tubes in the presence of 50 $\mu\text{g ml}^{-1}$ anti-E-selectin IgG (b), 50 $\mu\text{g ml}^{-1}$ of preimmune IgG (c), or 50 $\mu\text{g ml}^{-1}$ of anti-bovine P-selectin IgG (d). Capillary tubes were induced, fixed, and photographed as described in the legend to Fig. 1, except that cells were plated on 9-cm² dishes. The anti-E-selectin antiserum was raised against an 18-amino-acid peptide (SYHASTEMMTFEEARDYC; single-letter code) made by solid-phase synthesis (Berkeley Antibody Company, Richmond, CA). The purified peptide was coupled to keyhole limpet haemocyanin (Pierce) using the chemical crosslinking reagent *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (Pierce). Rabbits were immunized with the peptide conjugates by intradermal injection and boosted every 3 weeks. Anti-bovine P-selectin antiserum was prepared as described¹⁵. IgGs were purified by batch adsorption to Protein A-Sepharose Cl-4B (Sigma) and eluted with 0.1 M glycine, pH 2.3. Eluates were neutralized, dialysed against PBS, and sterile-filtered. Magnification, 50 $\times$.

BUD2 encodes a GTPase-activating protein for Bud1/Rsr1 necessary for proper bud-site selection in yeast

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CELLS of the budding yeast *Saccharomyces cerevisiae* bud at either axial or bipolar sites depending on their cell type¹. Bud-site selection directs both cell polarity and the cytoskeletal orientation during budding and is determined by at least five genes: *BUD1/RSR1*, *BUD2*, *BUD3*, *BUD4* and *BUD5*^{2–4}. Mutants defective in *BUD1*, *BUD2* or *BUD5* choose bud sites randomly. Bud1 protein (Bud1p) has sequence similarity to Ras², a small GTP-binding protein, and Bud5p is similar to Cdc25p (refs 4, 5), a GDP–GTP exchange factor⁶. Here we report that Bud2p is a GTPase-activating protein (GAP) for Bud1p with a sequence similar to the catalytic domain

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[illegible]

of rasGAPs, and that Bud2p purified from yeast stimulates GTP hydrolysis by Bud1p. Chromosomal deletion of *BUD2* causes a random budding pattern but no obvious growth defect. Overexpression of *BUD2* also causes a random budding pattern by wild-type cells¹¹.

BUD2 was cloned by complementation of the random budding phenotype of a *bud2* strain (see legend to Fig. 1). The predicted Bud2 protein contained 1,104 amino acids (Fig. 1b) with a calculated M_r of 127K. A region in the middle of the protein showed similarity to the carboxy-terminal portion of mammalian GAP (p120), which attenuates the activity of Ras by stimulating hydrolysis of Ras-bound GTP to GDP⁷. This region of mammalian GAP includes its catalytic domain. Several other proteins with GAP activity, including human neurofibro-

matosis type-I (NF1) protein and yeast Ira1 and Ira2 proteins, have also been characterized⁸⁻¹⁰. Comparison of the deduced amino-acid sequence of Bud2p with the catalytic domain of bovine GAP, NF1 and Ira2p revealed that Bud2p has 20.1% identity with bovine GAP over 299 amino acids, 21.5% identity with NF1 over 247 residues, and 19.5% identity with Ira2p over 231 residues (Fig. 1c).

The originally isolated *bud2* mutant exhibits a random budding pattern but has no growth defect³. To investigate further the role of Bud2p during vegetative growth, a strain deleted for *BUD2* was constructed, in which almost the entire *BUD2* coding sequence was replaced by a segment containing *LEU2*. Cells deleted for *BUD2* were able to germinate and grew well at 16 °, 25 °, 30 ° and 37 °C on both rich and minimal media. These *bud2*

c

Bud2	534	E L A K I D G T V S R I N O K N L D S K H V F N S L F R G N S I L T K S I E Q Y F F R V - G N E Y L S K A L S A I L K E I I E S
Ira2	1717	N A T H I - - V V A Q L I K N I K S S R P T D I L R N S C A T R S L S M L A R S K - G N E Y L I R T L Q P L L K K I I Q N
p120-GAP	764	K I E S L - - L C T I N D R I S M E D A T T L F R A T T L A S T L M E Q Y M K A T - A T Q F V H E A L K D S I L K I M E S
NF1	1251	H L L Y Q - - L W N M F S K V L A D S M Q T L F R G N S L A S K I M T F C E K V Y - G A T Y L Q K L L - D P L L R I V I T
Ser1	170	H L L L S - - F Q M V L T T F A T S D V L S L L R A N T P V E R M L T T Y T R R G P G Q A Y L R S I L Y Q C I N D V A I H
CONSENSUS		L L Φ E Φ L F R N S A S Φ Y G Y L L L I
Bud2	597	N K - - - - - S C E L D P A R V K E K D E - - - - - V K K R K I I A D N Y K R L Y S W V T K
Ira2	1778	R D - - - - - F F E I E K L K P - E D S D - - - - - A E R Q I E L F V K Y M N E L L E S
p120-GAP	825	K Q - - - - - S C E L S P S K L - E K N E D - - - - - V N T N L T H L L - N I L S E L V E K
NF1	1311	S S D W Q H V - - - - - S F E V D P T R L - E P S E S - - - - - L E N Q R N L L - Q M T E K F F H A
Ser1	232	P D L Q L D I H P L S V Y R Y L V N T G Q L S P S E D D N L L T N E E V S E F P A V K N A I Q E R S A Q L L - L L T K R F L D A
CONSENSUS		S E D P E E E L L Φ
Bud2	633	I W K R L Y A T S N D L P I E I R N V L K I F R Q K L E I I C I D D T L Q I I L N - - - G I S G L L F L R F F C P V I L N P K
Ira2	1811	I S N - - - - V S Y F P P F Y I C Q N - - - - - I Y K V A C E K Y F D H A I I - - - A A G S V F L R F F C P A L V P D
p120-GAP	859	I F M - - - - A S E I L P T R Y I Y G C - - - - - L Q K S V Q H K W T N T T M R - T R V V S G V F L R L I C P A I L N P R
NF1	1350	I I S - - - - S S E T P Q R S V H C - - - - - L Y Q V V S Q R T Q N S - - - - - I G A V S A M F L R F I N P A I V S P Y
Ser1	295	V L N - - - - I D E I P Y G I R W V K L - - - - - I R N L T N R L S I S D S T I C S L I G F E L R F V N P A I I S P Q
CONSENSUS		I S S Φ P P L R V C L F P G G F F L R F C P A I S P
Bud2	693	L E K Y V S Q N L N E T A R R N L T L I S K V L L N L S T L T Q F A N K E P W L M K M - N N F I D K R H N D L D Y I D K M T Q
Ira2	1863	S E N I E - I S H L S E K R F I S L K V I N I A G S E N F S R W P A L C S Q K - D F L E C S D R I F R F L A E L C R
p120-GAP	914	M E N I S D S S P I A A R L S L V K S V N L L V E F G A K E R Y - M E G V N P F I S N K H R M I M F L D E L G N
NF1	1402	E A G L K K P P R I E R G L K L M S K I L S I H V L E - T K E H - M R P F N D E V S N F D A A R R F L D I A S
Ser1	351	T S M L L S C S D N V R K L A T I K I I S V A N G T S S - T K T H L - D V S F Q P M L D E Y E E K V H N L R K E G N
CONSENSUS		I Φ D P R T L L Φ A K Φ Q N Φ A N F K E P M N F K Φ F L L
Bud2	756	K K L D F N S K I L N L S S T I S R P K L A I E Q T M L D D L P Q I - P Y L L
Ira2	1924	T D R T I D I Q V R T D P T P I A F D Y Q F H S F V Y L Y G L E V R R N V L
p120-GAP	977	V P - E L P D T T E H S R T D L S R D - L A A L H E I C V A - S D E L R T L S
NF1	1464	D C P T S D A V N H S L S F I S D G N V L A H R L L W N - Q E K I G Q Y L S
Ser1	413	V G D F S E A L E L D Q Y I A L S K K S L A E M T V N E I Y L T H E I I E
CONSENSUS		L A L Φ L

◀ FIG. 1 *BUD2* sequence and partial comparison of predicted amino-acid sequence of Bud2p with the catalytic domains of rasGAPs. a, Restriction map of the *BUD2* locus and location of the open reading frame. Hatched box indicates the position of a region similar to the catalytic domains of the rasGAPs. Restriction sites are as follows: B, *Bam*HI; Bg, *Bgl*II; R, *Eco*RI; Sc, *Scal*; M, *Msc*I; S, *Sal*I; X, *Xba*I. b, Nucleotide and predicted amino-acid sequences of *BUD2*. Amino-acid residues of a region similar to the catalytic domains of rasGAPs are underlined. The start and stop codons and the polyadenylation signal consensus are underlined. c, Comparison of predicted amino-acid sequence of Bud2p with the catalytic domains of rasGAPs. Numbers indicate amino-acid residue position. Residues identical to those of Bud2p are shown in white letters against black; residues common among at least three of the other GAPs are shaded; residues identical in three or more GAPs are shown as consensus. Φ, Hydrophobic residues. References to amino-acid sequences: Ira2²¹, p120-GAP⁷, NF1^{8,9}, Ser1²². METHODS. *BUD2* was cloned using a genomic DNA library in a low-copy number vector (YCp50) by complementation of the bud-site selection

defect of a *bud2* strain. About 10,000 transformants were screened microscopically. The complementing gene was confirmed to be the wild-type allele of the *bud2* mutation and not a suppressor by segregation analysis using the original *bud2* mutation and a mutation constructed *in vitro* in the cloned gene which was used to replace the chromosomal allele (see legend to Table 1). First, an *a/a* diploid formed by mating the mutant strain and the original *bud2* strain (IH2404) exhibited a random budding pattern. Second, a cross between the mutant and IH2404 yielded only random budding segregants in 24 tetrads. The dideoxy chain-termination method was used for sequencing M13 clones generated using restriction sites. The nucleotide sequences of both strands were determined completely by using several partially overlapping M13 clones and oligonucleotide primers. The predicted protein sequence was compared with the PIR/Dayhoff database using the FASTA program; significant homology was detected with the catalytic domain of rasGAPs. The *BUD2* nucleotide sequence is submitted to Genbank and has accession number L19162.

mutants showed a random budding pattern as determined by examination of microcolonies and Calcofluor staining of bud scars (Table 1). Ninety-one per cent of *bud2* mutant cells exhibited a non-axial budding pattern whereas only 10% of wild-type haploid cells exhibited a non-axial budding pattern. The effect of *BUD2* overexpression was examined by transforming wild-type strains with a high-copy number plasmid carrying *BUD2*. Of the haploid cells carrying this plasmid, 44% exhibited a non-axial pattern. In contrast, a high-copy plasmid carrying *BUD2* deleted for the presumed GAP catalytic domain did not affect the budding pattern (Table 1). Thus, both elimination and overproduction of Bud2p activity altered the budding pattern. Although possession of a plasmid overexpressing *BUD2* had little effect on cellular growth rate, 15–20% of cells exhibited elongated buds (data not shown).

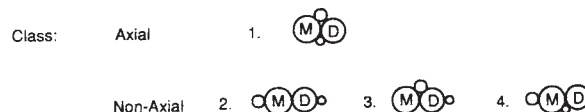
Two putative GTP-binding proteins involved in budding have been identified: Bud1p/Rsr1p, which is involved in bud-site selection^{2,3}, and Cdc42p, which is involved in polarity establishment¹¹. Bud1p is most similar to p21^{ap}, which is very closely related to Ras; Cdc42p is more similar to p21^{rho}. The similarity of the *bud2* and *bud1* mutant phenotypes (random budding pattern) and the sequence similarity of Bud2p to the rasGAP catalytic domain led to the suggestion that Bud1p may be a substrate for Bud2p. We confirmed that Bud1p binds GTP by protein blotting and incubation with [α -³²P]GTP (data not shown). To test whether Bud2p acts as a GAP towards Bud1p, epitope-tagged Bud1p was immunoprecipitated and allowed to bind to [α -³²P]GTP. Crude membrane fractions enriched for Bud2p (see legend to Fig. 2a) were added to determine the effect on the rate of GTP hydrolysis by Bud1p. Fractions from cells transformed with a plasmid carrying the *BUD2* gene on a high-copy plasmid enhanced the GTP hydrolysis rate by Bud1p more than 10-fold compared to the buffer control or to extract from a *bud2* deletion strain (Fig. 2a, lanes 2, 4 and 5). Fractions from the wild-type strain had only 2-fold higher activity than the control (Fig. 2a, lane 3). GAP activity was not observed in extracts lacking immunoprecipitated Bud1p (Fig. 2a, lane 1). We have also done the GAP assay with purified GST-Bud2 fusion protein and Bud1p (see legend to Fig. 2b). In this case, we measured the amount of [γ -³²P]GTP bound to Bud1p. GTP hydrolysis by Bud1p was strongly activated by GST-Bud2 fusion protein, whereas little GTP hydrolysis was observed by the GST control (Fig. 2b). These results indicate that Bud2p acts as a GAP for Bud1p.

Bud1p/Rsr1p has striking similarity to Ras², and Bud5p has similarity to Cdc25p^{4,5}, which is a guanine nucleotide exchange factor for Ras⁶. Our data show that *BUD2* encodes a GAP for Bud1p (Fig. 3a). First, the predicted amino-acid sequence of Bud2p has similarity to the catalytic domains of the rasGAP family of proteins. Second, both fractions enriched for Bud2p and GST-Bud2 fusion protein purified from yeast stimulated GTP hydrolysis activity by Bud1p. This GAP activity is important for proper bud-site selection: both *bud2* null mutations and overexpression of *BUD2* caused a random budding pattern. Cells expressing Bud1/Rsr1^{Val12}, which is believed to be equivalent to activated Ras^{Val19}, also display a random budding pattern¹². Ras^{Val19} is insensitive to GAP and causes a phenotype similar to that of *ira1* or *ira2* mutations^{10,13,14}. The ability of Bud1/Rsr1^{Val12} on a high-copy plasmid to support growth of a *RAS2(ts)* mutant is mimicked by wild-type Bud1/Rsr1 in a *bud2* *RAS2(ts)* mutant, which provides *in vivo* support for the conclusion that Bud2p is a GAP for Bud1p (ref. 15).

GTPase-activating proteins are viewed in two different contexts: first as a regulator of Ras or Ras relatives, and second as an effector for Ras^{16,17}. According to the first view, Bud2p functions solely to control the guanine nucleotide-bound state of Bud1p: it is responsible for converting the active, GTP-bound form to the inactive, GDP-bound form of Bud1p. According to the second view, Bud2p is an effector for the activated form of Bud1p. The observation that mutants lacking *BUD2* have the

TABLE 1 Budding pattern phenotypes of strains that lack or over-produce *BUD2*

Strain	Relevant genotype	Plasmid	Budding pattern	
			Axial	Non-axial
HP23	α <i>BUD2</i>		90	10
HP25	α <i>bud2</i> Δ :: <i>LEU2</i>		9	91
IH2390	α <i>BUD2</i>	YEp(<i>BUD2</i>)	56	44
IH2390	α <i>BUD2</i>	YEp(<i>CDA</i>)	87	13
IH2390	α <i>BUD2</i>	YEp24	89	11



The budding pattern of log-phase cells was determined as described in ref. 3. When mother cells (M) and daughter cells (D) are distinguished, four different patterns can be observed as indicated: class 1 is characteristic of the axial pattern; classes 2 and 3 are characteristic of the bipolar pattern; class 4 is an example of random budding pattern. Classes 2, 3 and 4 are grouped as non-axial pattern. About 400 clusters were analysed for each sample. Budding pattern is given as per cent of mother-daughter pairs exhibiting each pattern. Plasmid YEp(*BUD2*) contains the complete *BUD2* gene on the high-copy number vector YEp24; YEp(*CDA*) carries a deletion of a 612 base-pair (bp) *Scal*-*MscI* fragment which covers a region similar to the catalytic domain of GAP, resulting in deletion of residues 573–777 of Bud2p and insertion of a serine residue at the junction. Genotypes of strains used in this analysis are the following: HP25, *MATa ura3-52 lys2-801 ade2-101 his3 Δ 200 leu2 Δ 1 trp1 Δ 63 bud2 Δ ::*LEU2*; HP23, isogenic *BUD2* strain; IH2390, *MATa HMRa HMLa his4 trp1 ura3 can1 MAL2*. Methods. The *bud2* Δ ::*LEU2* mutation replaced almost the entire *BUD2* coding sequence with the *LEU2* gene and was constructed as follows. A *Bam*HI-*Sall* fragment containing *BUD2* and several hundred base pairs of flanking DNA on both ends was cloned into pUC19. A portion of this plasmid was amplified by the polymerase chain reaction using divergent primers that hybridized just upstream of the *BUD2* start codon and 30 nucleotides upstream of the stop codon. The resulting linear fragment was cleaved at a site introduced by the primers and circularized, yielding a plasmid that contained the *BUD2* flanking regions joined by a restriction site. A selective marker (*LEU2*) was then introduced into this site. A linear fragment containing the *LEU2* marker gene flanked by sequences adjacent to *BUD2* was used to replace the chromosomal *BUD2* gene by the one-step gene replacement method²⁰. Southern blotting of the genomic DNA confirmed that gene replacement had occurred by homologous recombination (data not shown). A *bud2* null mutation was generated in two different α/a diploid strain backgrounds. In both cases, the same phenotype was observed in meiotic progeny carrying the *bud2* mutation.*

same phenotype as mutants lacking *BUD1* is consistent with the idea that Bud2p is an effector for Bud1p. An alternative explanation is that Bud2p functions solely to govern the activity of Bud1p. In particular, one could argue that cycling of Bud1p from a GTP-bound state to a GDP-bound state is necessary for proper bud-site selection as suggested previously by Ruggieri *et al.*¹².

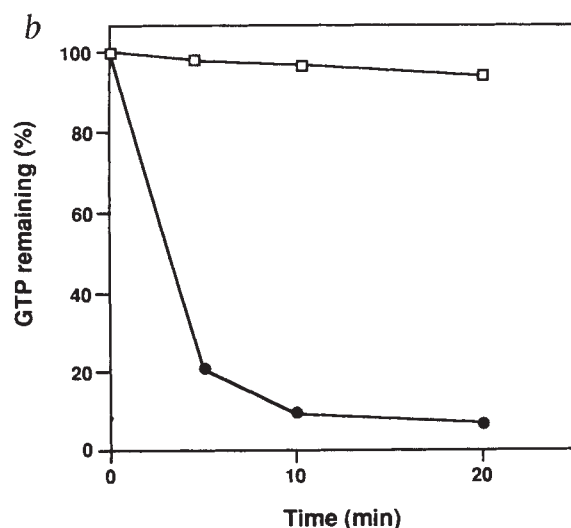
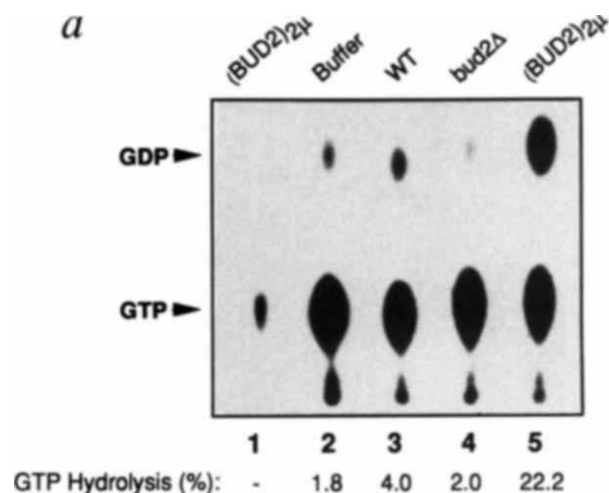
Bud1p, Bud2p, and Bud5p comprise a functional module involved in bud-site selection. How they function in this process is not known, but it is hypothesized that Bud1p interacts with one or more of the proteins (Cdc24p, Cdc42p and Bem1p) that are necessary for establishment of cell polarization and that Bud1p is involved in their localization or assembly³. By analogy to a model proposed for Sec4p function in vesicular transport^{18,19}, we propose that Bud1p-GTP may associate with Cdc24p (ref. 12), Cdc42p, or Bem1p, and thereby allow these

proteins to bind to the bud site (Fig. 3b). Hydrolysis of Bud1p-GTP stimulated by Bud2p (which might be located at the bud site or might travel with Bud1p) would allow recycling of Bud1p and further shuttling of proteins to the bud site. Determination of the cellular location of Bud proteins will be crucial to understand the mechanism of bud-site selection. In

particular, it will be important to know whether any of the Bud proteins colocalize with any of the proteins necessary for polarity establishment. It has recently been observed by immunofluorescence that Bud1 protein is not localized to the incipient budding site but rather is distributed over the cell surface in a punctate pattern (J.C. and J. Pringle, unpublished result). Thus,

FIG. 2 a, Identification of Bud2-dependent GAP activity for Bud1 protein. Haemagglutinin epitope-tagged Bud1p was immunoprecipitated with 12CA5 antibody²³ and was incubated with [α -³²P]GTP. The rate of GTP hydrolysis was determined in the absence (buffer control; lane 2) or presence of cell extracts from a wild-type strain (WT; lane 3), a *bud2* deletion strain (*bud2* Δ ; lane 4) or a wild-type strain carrying *BUD2* on a high copy plasmid YEp24 (*BUD2*_{2u}; lane 5). A negative control is shown in lane 1, in which immunoprecipitation was done with cell extract that did not express the epitope-tagged Bud1p. The GAP assay was done in this case with crude membrane fractions from the *Bud2p* overexpressing strain (*BUD2*_{2u}). Genotypes of strains used in this analysis are the following: *bud2* Δ (HP37), *MAT α ura3-52 his3- Δ 1 leu2 trp1 Δ 63 prb1-1122 pep4-3 prc1-407 bud2 Δ ::LEU2*; WT (HP16), isogenic *BUD2* strain. GTP hydrolysis (%) indicates the ratio of the amount of GDP to the sum of GDP and GTP for each reaction. **b**, GST-Bud2 fusion protein purified from yeast has GAP activity. GTPase activities of Bud1p with GST-Bud2 (●) and GST (□) purified from yeast are expressed as the percentage of [γ -³²P]GTP bound to Bud1p that is hydrolysed at each time point relative to initial [γ -³²P]GTP bound to Bud1p at time zero.

METHODS. **a**, The GAP assay was done by guanine nucleotide analysis by thin layer chromatography as described previously²⁴ with slight modifications. Bud2 protein was expressed as a fusion protein with a c-myc epitope at the N terminus or as a GST fusion protein in yeast to help localization of the protein. All procedures were at 4 °C unless specified otherwise. Yeast cells were resuspended in extraction buffer III (50 mM Tris-HCl, pH 7.4, 5 mM EGTA, 1 mM DTT, 0.2 mM PMSF, 2 mM benzamidine, 2 mM leupeptin) and lysed using glass beads in a bead-beater. A whole-cell extract was obtained after centrifugation at 500g for 5 min to remove cell debris and glass beads. The whole-cell extract was centrifuged at 10,000g for 15 min, generating pellet (P10) and supernatant (S10) fractions. The S10 fraction was further centrifuged at 100,000g for 60 min, generating pellet (P100) and supernatant (S100) fractions. The pellet fractions were resuspended in the same extraction buffer III, frozen in liquid nitrogen and then stored at -80 °C. Immunoblot analysis using monoclonal antibody 9E10 and polyclonal antibody against GST showed that Bud2p was present in both the particulate fractions P10 and P100 (data not shown), consistent with the previous report of a GAP activity for Bud1p/Rsr1p present in a membrane fraction²⁵. The sequence encoding a 9 amino-acid influenza HA epitope, YPYDVPDYA²⁶, was inserted in-frame within the *BUD1* gene after aspartic acid at residue 202 (ref. 2) by site-directed mutagenesis using synthetic oligonucleotides. A plasmid expressing this epitope-tagged Bud1p complemented a *bud1* deletion strain (H.-O. P., unpublished observations). Whole-cell extracts were prepared as described above but using extraction buffer I (50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 0.1 mM EDTA, 0.1% NP-40, 10% glycerol, and various protease inhibitors as above). HA-tagged Bud1p was immunoprecipitated using the whole-cell extract from cells equivalent to an absorbance of 50 at 600 nm (50 OD₆₀₀) with 12CA5 antibody and goat anti-mouse IgG-coupled agarose beads. After washing 5 times with extraction buffer I containing 500 mM NaCl and 0.5% Triton X100 and once with extraction buffer I, the immune complex was allowed to bind to [α -³²P]GTP at 30 °C. After extensive washing as above, the immune complex was mixed with an equal amount of pellet fraction (500 μ g of each P100 fraction, obtained from cells roughly equivalent to 40 OD₆₀₀). The control (lane 1), which was immunoprecipitated from extract of cells lacking HA-tagged Bud1p, received the same antibody and the same amount of [α -³²P]GTP, but retained little radioactivity after extensive washing. After incubation with pellet fractions prepared from each strain as listed in Fig. 2a or buffer alone for 15 min at 23 °C, the beads were washed again and nucleotides were eluted from Bud1p by incubating with 10 μ l of 1% SDS and 20 mM EDTA for 5 min at 65 °C. The nucleotides were loaded onto a PEI cellulose plate and chromatographed in 0.75 M potassium phosphate, pH 3.4. After autoradiography, the amount of each nucleotide was quantified using a Molecular Dynamics Phosphorimager. **b**, The entire protein-coding region of *BUD2* was fused with GST in-frame in a yeast/*E. coli* shuttle vector (pRD56) carrying GST under control of the *GAL* promoter. Yeast cells (HP16) carrying this plasmid



and the control plasmid (the vector without *BUD2* insert) were grown on medium containing 2% sucrose to log phase and induced with 2% galactose for 6–10 h. Yeast cells were resuspended in extraction buffer VI (20 mM HEPES, pH 7.8, 200 mM KCl, 5 mM EGTA, 1 mM DTT, 1% Tween 20, and various protease inhibitors as above) and lysed using glass beads in a bead beater. The whole cell extract was centrifuged at 10,000g for 15 min, and the supernatant was passed over a glutathione-sepharose 4B column (Pharmacia). After washing with extraction buffer VI containing 500 mM KCl and PBS, the proteins were eluted with 5 mM reduced glutathione (Sigma), 50 mM Tris-HCl, pH 8.0. To measure GTPase activity, Bud1p purified from Sf9 insect cells (gift from P. Polakis)²⁵ was preincubated with [γ -³²P]GTP in low MgCl₂. This GTP-loading reaction was stopped by increasing MgCl₂ (final 6.25 mM), and the GTPase activity was determined at 23 °C by measuring the loss of protein-bound radioactivity using a nitrocellulose filter-binding assay as described previously^{24,27}. The enzyme source was 40 ng GST-Bud2p or 100 ng GST protein partially purified from yeast.

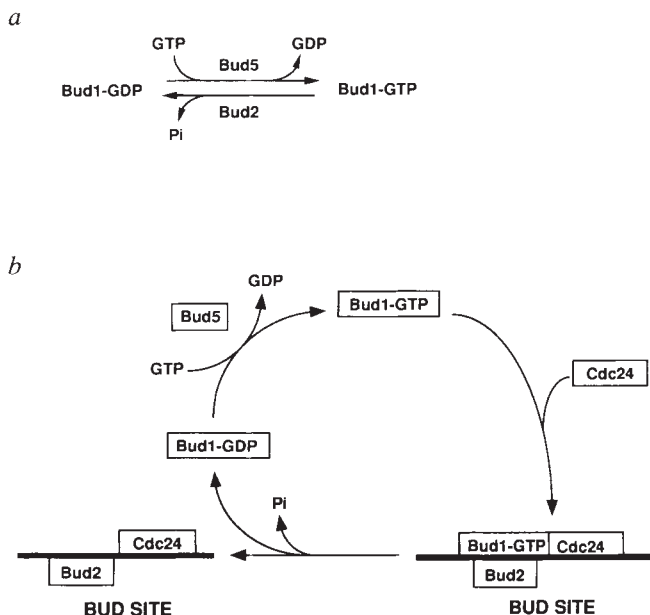


FIG. 3 *a*, Bud5p is proposed to be a guanine-nucleotide exchange protein for Bud1p. Bud2p functions as a GTPase-activating protein by catalysing GTP hydrolysis by Bud1p. *b*, A model for function of Bud1p in bud-site selection. In this scheme, Bud1p in its GTP-bound form associates with one of the proteins necessary for polarity establishment (such as Cdc24p, Cdc42p or Bem1p). Cdc24p is shown as an example. Bud1p can then deliver Cdc24p to the bud site, where Bud2p stimulates hydrolysis of Bud1p-GTP to Bud1p-GDP (see text). Another possibility is that Bud2p associates with Bud1p not only to catalyse GTP hydrolysis by Bud1p but also to assist in binding of Cdc24p to Bud1p.

it appears that the position of Bud1p is not sufficient to direct positioning of bud-formation proteins. Its activity may be determined by colocalization of its exchange protein (presumed to be Bud5p) or its GAP (Bud2p), which may be crucial to direct positioning of other proteins. □

Destabilization of tracts of simple repetitive DNA in yeast by mutations affecting DNA mismatch repair

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THE genomes of all eukaryotes contain tracts of DNA in which a single base or a small number of bases is repeated. Expansions of such tracts have been associated with several human disorders including the fragile X syndrome¹. In addition, simple repeats are unstable in certain forms of colorectal cancer, suggesting a defect in DNA replication or repair²⁻⁴. We show here that mutations in any three yeast genes involved in DNA mismatch repair (*PMS1*, *MLH1* and *MSH2*) lead to 100- to 700-fold increases in tract instability, whereas mutations that eliminate the proof-reading function of DNA polymerases have little effect. The meiotic stability of the tracts is similar to the mitotic stability. These results suggest that tract instability is associated with DNA polymerases slipping during replication, and that some types of colorectal cancer may reflect mutations in genes involved in DNA mismatch repair.

Tracts of simple repetitive DNA change length at frequencies that are considerably higher than expected for 'standard' point mutations. One mechanism that has been proposed to account for such alterations is unequal crossing-over (Fig. 1*a*) (crossing-over between tracts misaligned by an integral number of repeats⁵). An alternative mechanism is DNA polymerase slippage⁶ (Fig. 1*b*): during replication of the tract, the primer and template strand transiently dissociate and then reassociate in a misaligned configuration. If the unpaired bases are in the primer strand, continued synthesis results in an elongation of the tract whereas unpaired bases in the template strand results in a deletion. One way of determining which of these mechanisms is most important in controlling tract stability is to examine the effects of mutations in various defined genes. Mutations affecting recombination in *Escherichia coli* (*recA*)⁷ and yeast (*rad52*) (ref. 8) do not affect tract stability, although *E. coli* strains with *mutL* and *mutS* mutations (leading to defective mismatch repair) had 13-fold elevated frequencies of tract instability⁷. In addition, a mutation in a yeast *MutL* homologue *pms1* stimulates reversion of a *hom3* allele caused by a single base-pair frameshift mutation⁹.

The most common simple repeat in most eukaryotes is poly(GT)₁₀₋₃₀ (ref. 10). We examined the genetic control of the stability of poly(GT) tracts using two types of centromere-containing plasmids⁸. The plasmid pSH31 contained a yeast promoter fused to a β -galactosidase gene that contained a 29-base pair (bp) (out-of-frame) poly(GT) tract in the coding sequence; alterations in tract length that resulted in an in-frame insertion were detected as blue yeast colonies on X-GAL plates. The plasmid pSH91 contained an in-frame insertion of poly(GT) (33 bp) in the coding sequence of the yeast *URA3* gene; alterations in tract length resulting in an out-of-frame insertion resulted in colonies on plates containing 5-fluoro-uracil, a drug that selects against *Ura*⁺ cells¹¹.

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Received 8 March; accepted 2 July 1993.

- Drubin, D. *Cell* **65**, 1093-1096 (1991).
- Bender, A. & Pringle, J. *Proc. natn. Acad. Sci. U.S.A.* **86**, 9976-9980 (1989).
- Chant, J. & Herskowitz, I. *Cell* **65**, 1203-1212 (1991).
- Chant, J., Corrado, K., Pringle, J. & Herskowitz, I. *Cell* **65**, 1213-1224 (1991).
- Powers, S., Gonzales, E., Christensen, T., Cubert, J. & Broek, D. *Cell* **65**, 1225-1231 (1991).
- Jones, S., Vignais, M. L. & Broach, J. R. *Molec. cell. Biol.* **11**, 2641-2646 (1991).
- Vogel, U. S. *et al.* *Nature* **335**, 90-93 (1988).
- Xu, G. *et al.* *Cell* **63**, 835-841 (1990).
- Ballester, R. *et al.* *Cell* **63**, 851-859 (1990).
- Tanaka, K. *et al.* *Cell* **60**, 803-807 (1990).
- Johnson, D. I. & Pringle, J. R. *J. Cell Biol.* **111**, 143-152 (1990).
- Ruggieri, R. *et al.* *Molec. cell. Biol.* **12**, 758-766 (1992).
- Broek, D. *et al.* *Cell* **41**, 763-769 (1985).
- Tanaka, K., Matsumoto, K. & Toh-e, A. *Molec. cell. Biol.* **9**, 757-768 (1989).
- Bender, A. *Proc. natn. Acad. Sci. U.S.A.* (in the press).
- McCormick, F. *Cell* **56**, 5-8 (1989).
- Hall, A. *Cell* **61**, 921-923 (1990).
- Bourne, H. R., Sanders, D. A. & McCormick, F. *Nature* **348**, 125-132 (1990).
- Walworth, N. C., Goud, B., Kabaceni, A. K. & Novick, P. *J. EMBO J.* **8**, 1685-1693 (1989).
- Rothstein, R. *Meth. Enzym.* **101**, 202-209 (1983).
- Tanaka, K. *et al.* *Molec. cell. Biol.* **10**, 4303-4313 (1990).
- Wang, Y., Boguski, M., Riggs, M., Rodgers, L. & Wigler, M. *Cell Reg.* **2**, 453-465 (1991).
- Field, J. *et al.* *Molec. cell. Biol.* **9**, 2159-2165 (1988).
- Tanaka, K., Lin, B. K., Wood, D. R. & Tamarit, F. *Proc. natn. Acad. Sci. U.S.A.* **88**, 468-472 (1991).
- McCabe, P., Haubruck, H., Polakis, P., McCormick, F. & Innis, M. A. *Molec. cell. Biol.* **12**, 4084-4092 (1992).
- Kolodziej, P. & Young, R. A. *Meth. Enzym.* **194**, 508-519 (1991).
- Polakis, P. G., Rubinfeld, B., Evans, T. & McCormick, F. *Proc. natn. Acad. Sci. U.S.A.* **88**, 239-243 (1991).

ACKNOWLEDGEMENTS. We thank members of the Herskowitz laboratory for discussion and assistance during this project, H. Bourne for comments on the manuscript, F. McCormick, G. Bollag and M. Ruggieri for discussion, P. Polakis for Bud1 protein, D. Kellogg for GST antibody, G. Ramsay for monoclonal antibody 9E10 and R. Deshaies for a plasmid. This work was supported by research grants from the American Cancer Society and from the NIH to I.H. J.C. was supported by fellowships from the Medical Research Council of Canada, the Lucille P. Markey Charitable Trust, and the Weingart Foundation.

Three mutations affecting mismatch repair in yeast were examined, *pms1* and *mlh1* (homologues of *MutL* in *Salmonella*¹²), and *msh2* (a homologue of *MutS* of *Salmonella*¹³). The gene *MLH1* was isolated using the polymerase chain reaction (PCR) to detect sequences homologous to *PMS1* (T.A.P., D.-M. Christie and R.M.L., manuscript in preparation). All three mutations

TABLE 1 Rates of alteration in lengths of poly(GT) tracts in yeast strains with mutations affecting DNA mismatch repair

Strain	Relevant genotype	Tract location	Rate of tract alterations	Rate relative to wild type
MS85	Wild type	pSH31	3.1×10^{-6}	1
MS57	<i>pms1</i>	pSH31	2.4×10^{-3}	774
MS90	<i>mlh1</i>	pSH31	1.7×10^{-3}	548
MS94	<i>msh2</i>	pSH31	1.1×10^{-3}	355
MS97	<i>pms1 mlh1</i>	pSH31	1.7×10^{-3}	548
MS86	<i>pol2</i>	pSH31	2.2×10^{-6}	0.7
MS87	<i>pol3</i>	pSH31	3.4×10^{-5}	11
MS96	Wild type	pSH91	1.6×10^{-5}	1
MS70	<i>pms1</i>	pSH91	4.0×10^{-3}	250
MS91	<i>mlh1</i>	pSH91	2.6×10^{-3}	163
MS79	<i>pol2</i>	pSH91	6.0×10^{-6}	0.4
MS80	<i>pol3</i>	pSH91	7.6×10^{-5}	5
SH75	Wild type	Chromosome	1.1×10^{-5}	1
MS89	<i>pms1</i>	Chromosome	8.5×10^{-4}	77

All strains used in this study, except SH75 and MS89, were derived from strain AMY125 (*a ade5-1 his7-2 leu2-3,112 trp1-289 ura3-52*) by transformation. Most strains contained either plasmid pSH31 or pSH91 to monitor tract stability (as described below). Control strains MS85 and MS96 were derived from a *Leu*⁺ derivative of AMY125 constructed by transformation with the plasmid CV9 (ref. 19). MS57 and MS70 were derived from AMY101 (*LEU2* insertion replacing the *PMS1* gene in AMY125), and MS90 and MS91 were derived from AMY125Δ*mlh1* (AMY125 with a *LEU2* insertion replacing a portion of the coding sequence of *MLH1*). MS94 was derived by transforming AMY125 with pII-2-Tn10LUK7-7 resulting in a *URA3* insertion in *MSH2*. MS97 was derived from AMY101Δ*mlh1* (AMY125 with a *LEU2* insertion in *PMS1* and a *URA3* insertion in *MLH1*). A *Leu*⁺ derivative of XAM8A (AMY125 with *pol2-4* mutation)¹⁵ was obtained by transformation with CV9; MS86 and MS79 were derived from this strain. MS80 and MS87 were constructed using a *Leu*⁺ derivative of AMY126 (AMY125 with *pol3-01*)¹⁵ obtained by transformation of AMY126 with CV9. The construction of SH75 (*a trp1 arg4-17 his4-Sal tyr7-1 ade6 ura3 lys2::* out-of-frame β -galactosidase gene) has been reported previously⁸. The length of the poly(GT) tract in SH75 is 29 bp. MS89 was obtained by transforming SH75 with pJH523, resulting in a deletion of *PMS1*. In strains SH75, MS89, and all strains with pSH31, alterations in tract length were detected by plating cells on medium containing X-GAL (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside). As these strains contained β -galactosidase genes with out-of-frame insertions of poly(GT) (29-bp tracts), cells with these genes formed white colonies on X-GAL plates unless the tracts altered in size to generate an in-frame insertion. Rates were determined by measuring the frequency of blue colonies in a large number of independent cultures (more than 20), and converting these frequencies to rates using standard methods^{20,21}. The plasmid pSH91 contains an in-frame insertion of poly(GT) (33 bp) in *URA3*; this plasmid is identical to pSH36 (ref. 8) except for the size of the tract. Alterations in tract size resulting in out-of-frame insertions were detected by plating cells on medium containing 5-fluoro-uracil which selects for *Ura*⁺ cells¹¹.

have a similar mutator phenotype and lead to high levels of post-meiotic segregation (resulting from failure to repair mismatches formed during meiotic recombination). In two different genetic backgrounds using two different plasmid assay systems, all three mutations lead to 100- to 700-fold elevated levels of tract instability (Table 1). In addition, a poly(GT) tract located within the chromosome has a 77-fold elevated rate of instability in a *pms1* strain (comparison of MS89 and SH75); we do not know whether the lower rate of tract instability for the chromosomal insertion relative to the plasmid insertion represents a chromosome-plasmid difference or an effect of flanking DNA sequences. A *pms1 mlh1* double mutant strain (MS97) has about the same instability as that observed for the single mutants, indicating that these gene products may act in the same pathway. Similar observations have been made for the mutator and post-meiotic segregation phenotypes of *pms1 mlh1* double mutants (T.A.P., D.-M. Christie and R.M.L., manuscript in preparation).

As the *pms1* mutation stimulates mitotic recombination only slightly (fivefold)⁹, we suggest that dramatic elevation of tract instability in repair-defective mutants reflects the failure to remove the mismatched bases present after a DNA polymerase slippage event (Fig. 1b). We suggest that, in wild-type cells, these mismatches are usually corrected by local excision of bases from the newly synthesized strand, followed by repair synthesis using the 'old' strand as a template.

We sequenced the altered tracts from a number of strains with a phenotype indicating an alteration in poly(GT) tract length (Table 2). In the wild-type strain MS96, 21 of the 24 plasmids analysed had altered tract lengths; the three strains with unaltered tracts presumably had a *ura3* mutation elsewhere in the coding sequence. Seventeen of the 21 altered tracts had deletions or insertions of 1 or 2 repeat units, and 4 had more extensive changes; the small frameshifts had a significant ($P=0.03$) bias favouring insertions. In *pms1*, *mlh1* or *msh2* mutant strains, only deletions or insertions of one or two repeats were observed. The lack of tracts with more extensive alterations in the mutant strain (MS70) compared to the wild-type strain (MS96) is statistically significant by the Fisher exact test ($P=0.013$). The simplest interpretation of this result is that the yeast *PMS1/MSH2/MLH1* mismatch repair system fails to recognize efficiently insertions or deletions that exceed 4 bp in size. In *E. coli*, the *mutHLS* system efficiently recognizes heterologies of 3 bp or less, recognizes less efficiently a 4 bp heterology, and fails to recognize 5 bp heterologies¹⁴.

Yeast contains two DNA polymerases: δ (encoded by *POL3*) and ϵ (encoded by *POL2*) that have 3'-5' exonuclease ('proof-reading') activities, and strains with mutations in the nuclease portion of these genes have frequencies of mutations elevated

TABLE 2 Sequence analysis of altered poly(GT) tracts

Strain	Relevant genotype	Tract location	Number of tracts sequenced	Number of tracts with additions (+) or deletions (−) of base pairs				
				+4	+2	−2	−4	Other
MS96	Wild type	pSH91	24	1	14	2		4 (−10,−10,−14,−16)
MS70	<i>pms1</i>	pSH91	36	2	19	14	1	
MS91	<i>mlh1</i>	pSH91	15	2	7	6		
MS94	<i>msh2</i>	pSH31	11		5	5		
MS79	<i>pol2</i>	pSH91	15		6	2	1	2 (+16,−20)
MS80	<i>pol3</i>	pSH91	29		15	6		
SH75	Wild type	Chromosome	10	1		8		1 (+10)
MS89	<i>pms1</i>	Chromosome	11			11		

Plasmid DNA was isolated from yeast strains with a potential alteration in poly(GT) tract length, detected as *Ura*⁺ (5-fluoro-uracil resistant) colonies in strains with pSH91 or as blue colonies on X-GAL plates in the strain with pSH31. Plasmid DNA was transformed into *E. coli* and the plasmid-borne tracts were sequenced by standard methods. Although most of these plasmids had tracts of altered length, some had the original tract length, indicating that the altered phenotype reflected a mutation elsewhere in the gene. In strains SH75 and MS89, alterations in tract length were detected by the formation of blue colonies on X-GAL plates. Because the poly(GT) tracts in this strain were inserted into the chromosome, we sequenced the DNA fragment resulting from asymmetric PCR amplifications of this region of the chromosome, as described previously for SH75 (ref. 8).

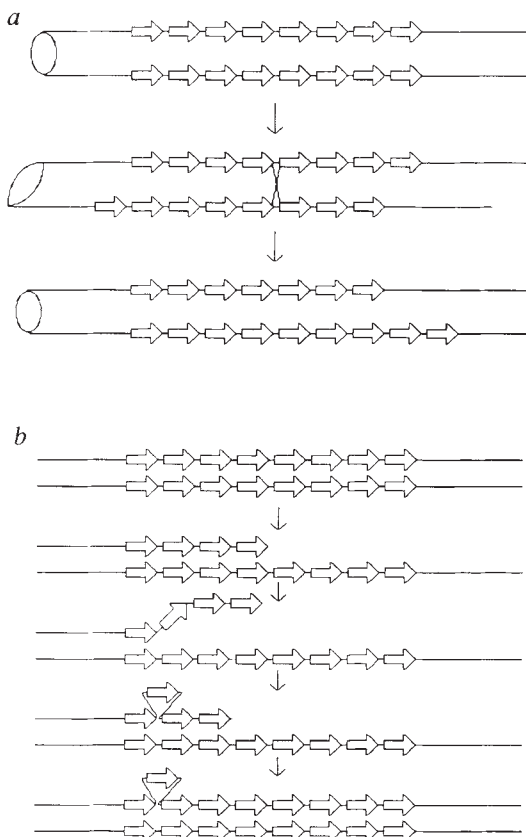


FIG. 1 Mechanisms that cause tract instability. *a*, Unequal crossing-over⁵. Crossovers between misaligned repeats (each repeat indicated by an arrow) result in a deletion from one tract and an addition to another. Gene conversion events could cause non-reciprocal alterations in tract length. *b*, DNA polymerase slippage⁶. During DNA replication (or repair synthesis), one strand transiently dissociates from the other, and then reanneals in a misaligned configuration. If the mismatched bases are located in the primer strand (as shown), continued elongation results in an increased tract length. If the unpaired bases are located in the template strand, one of the tracts would contain a deletion.

several hundredfold¹⁵⁻¹⁷. Although there seems to be a small effect of the *pol3* mutation (5- to 10-fold), neither *pol2* nor *pol3* mutations affect tract stability as dramatically as mutations that reduce mismatch repair (Table 1). Sequence of poly(GT) tracts of plasmids derived from *pol2* and *pol3* strains (Table 2) indicates that the types of alterations are similar to those observed in wild-type strains, although a somewhat higher fraction of the plasmids appear to have *ura3* mutations that do not involve the poly(GT) tract (27% versus 12%). One interpretation of the relatively small effect of the mutations in *pol2* and *pol3* is that the mispaired bases are not located at the end of the elongating strand, but several base pairs away, and are undetected by the polymerase proof-reading function. Alternatively, the proof-reading function may be insensitive to heterologies of more than one base pair.

We also compared mitotic and meiotic rates of tract stability. The frequency of recombination is elevated about 100- to 1,000-fold in meiosis relative to mitosis¹⁸. A diploid strain MS48 was constructed (genotype: *a/a trp1/trp1 ura3-52/ura3-x lys2/LYS2 ade2/ADE2 ARG4/arg4 TYR7/tyr7 ADE6/ade6*) containing pSH31 (out-of-frame insertion of the β -galactosidase gene). The average rate of tract alterations (four experiments) was 1.3×10^{-5} per mitotic cell division, and 3.2×10^{-5} per meiotic cell division. Completion of meiosis in MS48 was monitored by spore formation. We found that meiosis resulted in increased (30-fold) levels of recombination at the heteroallelic *ura3* locus without an increase in tract instability, further supporting the conclusion that tract instability does not reflect normal recombination processes. As reciprocal crossovers involving pSH31 would produce an unstable dicentric plasmid, our conclusions concern only non-reciprocal recombination events (gene conversions).

The results show that the instability of poly(GT) tracts in

yeast is increased by mutations in the mismatch repair genes *PMS1*, *MLH1* and *MSH2*, but is relatively unaffected by mutations affecting the proofreading functions of DNA polymerases PolII or PolIII. These results indicate that DNA polymerase *in vivo* has a very high rate of slippage on templates containing simple repeats, but most of these errors are corrected by cellular mismatch repair systems. Thus, the instability of simple repeats observed for some human disease may be a consequence of either an increased rate of DNA polymerase slippage or a decreased efficiency of mismatch repair. Whether slippage events lead to a net increase or decrease in tract length may also be affected by either the position of the unpaired bases (loop formed in template or primer strand) or the frequency with which different types of errors (mismatched bases in primer or template strand) are corrected. Finally, we note that the phenotype of the mutation involved in one type of familial colorectal cancer (decreased stability of simple repeats)²⁻⁴ is that predicted for a mutation affecting DNA mismatch correction. Such a mutation could represent a functional homologue of *PMS1*, *MLH1* or *MSH2* or another component of the mismatch repair system (for example, a DNA helicase or single-strand binding protein). □

Received 16 June; accepted 10 August 1993.

1. Caskey, C. T., Pizzuti, A., Ying-Hui, F., Fenwick, R. G. & Nelson, D. L. *Science* **256**, 784-789 (1992).
2. Aaltonen, L. A. et al. *Science* **260**, 812-818 (1993).
3. Thibodeau, S. N., Bren, G. & Schaid, D. *Science* **260**, 818-821 (1993).
4. Ionov, Y., Peinado, M. A., Malkhosyan, S., Shibata, D. & Perucho, M. *Nature* **363**, 558-561 (1993).
5. Smith, G. P. *Cold Spring Harb. Symp. quant. Biol.* **38**, 507-513 (1973).
6. Streisinger, G. et al. *Cold Spring Harb. Symp. quant. Biol.* **31**, 77-84 (1966).
7. Levinson, G. & Gutman, G. A. *Nucleic Acids Res.* **15**, 5323-5338 (1987).
8. Henderson, S. T. & Petes, T. D. *Molec. cell. Biol.* **12**, 2749-2757 (1992).
9. Williamson, M. S., Game, J. C. & Fogel, S. *Genetics* **110**, 609-646 (1985).
10. Hamada, H., Petrino, M. G. & Kakunaga, T. *Proc. natn. Acad. Sci. U.S.A.* **79**, 6465-6469 (1982).
11. Boeke, J. D., Lacroute, F. & Fink, G. *Molec. Gen. Genet.* **197**, 345 (1984).
12. Kramer, W., Kramer, B., Williamson, M. S. & Fogel, S. *J. Bact.* **171**, 5339-5346 (1989).
13. Reenan, R. A. G. & Kolodner, R. D. *Genetics* **132**, 963-973 (1992).
14. Parker, B. O. & Marinus, M. G. *Proc. natn. Acad. Sci. U.S.A.* **89**, 1730-1734 (1992).
15. Morrison, A., Bell, J. B., Kunkel, T. A. & Sugino, A. *Proc. natn. Acad. Sci. U.S.A.* **88**, 9473-9477 (1991).
16. Morrison, A., Johnson, A. L., Johnston, L. H. & Sugino, A. *EMBO J.* **12**, 1467-1473 (1993).
17. Simon, M., Giot, L. & Faye, G. *EMBO J.* **10**, 2165-2170 (1991).
18. Petes, T. D., Malone, R. E. & Symington, L. S. in *The Molecular and Cellular Biology of the Yeast Saccharomyces* Vol. 1 (eds Broach, J. R., Pringle, J. R. & Jones, E. W.) 407-521 (Cold Spring Harbor Laboratory Press, New York, 1991).
19. Klein, H. L. & Petes, T. D. *Molec. cell. Biol.* **4**, 329-339 (1984).
20. Luria, S. E. & Delbruck, M. *Genetics* **28**, 491-511 (1943).
21. Lea, D. E. & Coulson, C. A. *J. Genet.* **49**, 264-285 (1949).

ACKNOWLEDGEMENTS. We thank E. Alani, J. Haber, S. Henderson, R. Kolodner, A. Sugino and A. Morrison for providing some of the plasmids and strains. The research was supported by grants from the American Cancer Society (T.D.P.) and the NIH (R.M.L.).

Structure of a new nucleic-acid-binding motif in eukaryotic transcriptional elongation factor TFIIS

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TRANSCRIPTIONAL elongation involves dynamic interactions among RNA polymerase and single-stranded and double-stranded nucleic acids in the ternary complex¹⁻⁴. In prokaryotes its regulation provides an important mechanism of genetic control¹. Analogous eukaryotic mechanisms are not well understood⁵, but may control expression of proto-oncogenes^{6,7} and viruses, including the human immunodeficiency virus HIV-1 (ref. 8). The highly conserved eukaryotic transcriptional elongation factor TFIIS⁹ enables RNA polymerase II (RNAPII) to read through pause or termination sites, nucleosomes and sequence-specific DNA-binding proteins¹⁰⁻¹⁴. Two distinct domains of human TFIIS, which bind RNAPII and nucleic acids, regulate read-through¹⁰ and possibly nascent transcript cleavage¹¹⁻¹⁵. Here we describe the three-dimensional NMR¹⁶ structure of a Cys₄ nucleic-acid-binding domain from human TFIIS^{9,10}. Unlike previously characterized zinc modules¹⁷⁻²¹, which contain an α -helix, this structure consists of a three-stranded β -sheet. Analogous Cys₄ structural motifs may occur in other proteins involved in DNA or RNA transactions²²⁻²⁴, including RNAPII itself²⁵. This new structure, designated the Zn ribbon, extends the repertoire of Zn-mediated peptide architectures²⁶ and highlights the growing recognition of the β -sheet as a motif of nucleic-acid recognition^{27,28}.

The nucleic-acid-binding domain of TFIIS (C-terminal residues 231-280) contains a novel Cys₄ Zn²⁺-binding site that is required for activity¹⁰. In its absence, TFIIS fragments retain RNAPII binding but inhibit (rather than stimulate) elongation. Nucleic-acid binding is not exhibited by the intact protein but is 'unmasked' by deletion of the RNAPII-binding domain. Unmasking of a cryptic nucleic-acid-binding domain by deletion of adjoining sequences has also been described in sigma factor σ^{70} , a subunit of *Escherichia coli* RNAP required for specific initiation²⁹; occluding sequences in the native protein presumably reorganize on binding to RNAP core subunits. An analogous model is proposed for TFIIS¹⁰ and supported by identification of point mutations in the C-terminal domain that reduce factor-stimulated transcriptional elongation and weaken nucleic-acid binding by the isolated Cys₄ domain (C.-J.J. and K.A., unpublished results). Assembly-dependent exposure of protein surfaces may limit nonspecific nucleic-acid binding by components of the basal transcriptional machinery^{10,29}.

When the TFIIS nucleic-acid-binding domain was expressed in *E. coli* with and without N-terminal His₆ sequences, nucleic-acid binding was similar in each case (Fig. 1). The peptides were monomeric, contained one Zn²⁺ ion and gave similar circular dichroism and ¹H-nuclear magnetic resonance (NMR) spectra (see Fig. 1 caption). The His₆ extended domain was investigated by three-dimensional NMR¹⁶, as described elsewhere²⁴. Its structure (peptide residues 5-50) was calculated by distance-geometry/restrained molecular dynamics (DG/RMD) based on 934 restraints (Fig. 2). The structure is elongated: a disordered loop is observed at one end (residues 27-34) and an internal Zn-binding site at the other (red arrow in Fig. 2b). The structure is

similar to that of other Zn modules¹⁷⁻²⁰ in that Zn²⁺ coordination stabilizes a globular mini-domain with well defined secondary structure, nonpolar interior and polar or charged surface. The topology is otherwise different from previously characterized Zn modules^{17,21,26}, defining a new motif. Its central feature is a three-stranded antiparallel β -sheet (Fig. 2b); β -strands consist of residues 20-25, 35-40 and 45-48. Each C-X₂-C element defines a β -turn. Figure 2d shows the DG/RMD ensemble; representative side chains are labelled in Fig. 2c. Inspection of sequence databases identifies analogous motifs in the 16K subunit of yeast RNAPII (ref. 25), the RNAPII transcriptional initiation factor TFIIE (ref. 22), T-phage DNA primases²³ and other proteins involved in RNA or DNA transactions²⁴.

The α -helix and β -sheet provide complementary mechanisms for nucleic-acid binding^{27,28}. Such mechanisms are well characterized in sequence-specific recognition of double-stranded DNA. Less well understood are dynamic protein-nucleic-acid interactions involving non-canonical nucleic-acid structures, as must occur as reaction intermediates in transcription, replication and recombination. Although its mechanism of nucleic-acid

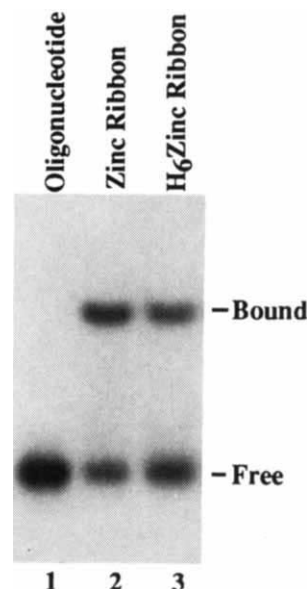


FIG. 1 Binding of single-stranded DNA (ssDNA; 45 bases) by the isolated Zn ribbon and His₆ extended Zn ribbon is demonstrated by gel mobility-shift assay. Lane 1, 5' ³²P-labelled oligodeoxynucleotide alone; lane 2, ³²P-labelled ssDNA and Zn ribbon; and lane 3, ³²P-labelled ssDNA and His₆ extended Zn ribbon. The ssDNA sequence was 5'-GGCCCGTCCCCCTGTGCCCTCTTTGTTGCAATTCCTCTGGTTC-3' (designated D1; ref. 10).

METHODS. The domain (TFIIS residues 231-280; refs 9,10) was expressed as a 55-residue peptide containing 5 additional residues at the N terminus (MARAP; numbered -4 to 0) and as a 61-residue His₆ fusion peptide²⁴ containing 11 additional residues at the N terminus (MRHHHHHPMA; numbered -10 to 50). Peptide residues 1-50 correspond to protein residues 231-250 (ref. 9). The 55-residue peptide was purified as a 1:1 Zn²⁺-complex by the method described for intact protein¹⁰; the His₆ extended peptide was purified as a 1:1 Zn²⁺ complex by nickel-affinity chromatography (Invitrogen, San Diego)²⁴. Thiolate coordination of one Zn²⁺ is demonstrated in the intact protein and fragments by atomic absorption and extended X-ray fine structure (EXAFS)¹⁰. In gel mobility-shift assays, 2 nM ³²P-5'-labelled oligodeoxynucleotide was incubated with 200 nM peptide at room temperature for 30 min in 200 mM KCl, 2 mM MgCl₂, 1 mM dithiothreitol, 25 mM HEPES (pH 7.5), 0.1 mM ethylenediaminetetraacetic acid, 100 µg ml⁻¹ bovine serum albumin, 0.05% NP-40 and 10% glycerol (vol/vol).

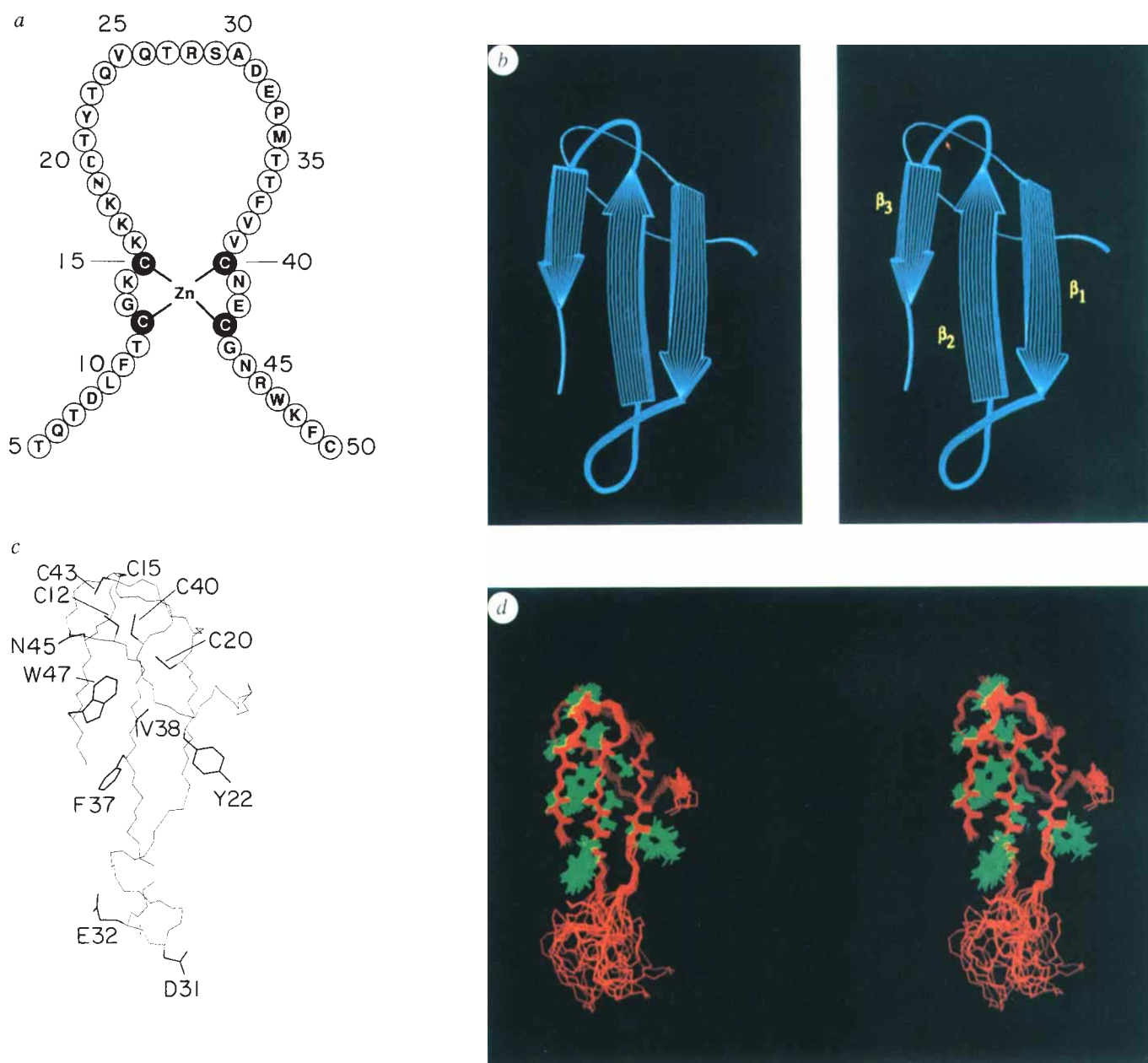


FIG. 2 *a*, Sequence of TFIIIS domain^{9,10} (single-letter amino-acid code); cysteines in the Zn-binding site are shown in black. *b*, Ribbon model (stereo pair). The position of Zn is indicated by a red arrow; β -strands are labelled β_1 – β_3 . The model was generated using the program *Ribbon* (J. P. Priestle). *c*, DG/RMD model with selected side chains labelled. *d*, DG/RMD ensemble (stereo pair) aligned as in *c*. The main chain is shown in red and selected side chains in green. The N-terminal S_{γ} in each C–X₂–C element (C12 and C40) forms bifurcating sulphur–amide hydrogen bonds²⁴; in the Zn-finger such bonds involve both S_{γ} atoms^{17,19,30}. The interior of the TFIIIS domain contains edge-to-face aromatic packing (involving W47, F10 and F49) reminiscent of that in the Zn-finger^{17,19}. DNA or RNA contact residues have not been determined; addition of ssDNA (5'-GAGATGACTCATCTC) leads to broadening of resonances in the β -strands but not in the N-terminal extension or central loop.

METHODS. NMR spectra (three-dimensional TOCSY–NOESY, ¹⁵N-edited three-dimensional NOESY and standard two-dimensional experiments) were obtained at 500 MHz in 50 mM deuterated Tris–HCl (pH 6.0) and 10 mM deuterated dithiothreitol at a peptide:Zn stoichiometry of 1:1 as described^{24,30}. No structural information was obtained for residues N-terminal to T5, which seem to be disordered. The structure of the

central loop (residues 27–34) was also not well defined. DG/RMD structures (residues 5–50) were calculated using programs *DGII* (T. A. Havel) and *X-PLOR* (A. T. Brünger) based on 637 upper-bound distance restraints (162 intrasite, 216 sequential, 48 medium-range and 211 long-range), 236 lower-bound restraints, 35 dihedral angle restraints (20° and 15°), 16 distance restraints related to hydrogen bonds (two restraints per hydrogen bond) and 10 distance restraints related to Zn coordination. Hydrogen-bond restraints were introduced based on slowly exchanging amide resonances in D₂O (ref. 30). Average violations of upper-bound distance and dihedral restraints were 0.028 Å and 1.2°, respectively. Excluding disordered regions, root-mean-square deviations were 0.40 Å and 0.89 Å for backbone and side-chain atoms, respectively. Assignment of ligands: Although the peptide contains six cysteines, only four are invariant among TFIIIS homologues (Cys 12, Cys 15, Cys 40 and Cys 43)⁹; mutation of the two non-conserved cysteines (Cys 20→Val and Cys 50→Ser) are functionally conservative and do not affect Zn²⁺ binding (C.-J.J. and K.A., unpublished results). In DG/RMD structures calculated in the absence of Zn-related restraints, the conserved cysteines define a unique tetrahedral Zn²⁺-binding site. Inclusion of Cys 20 or Cys 50 in potential Zn-binding sites leads to significant restraint violations²⁴.

binding is not known, the TFIIS domain binds in gel-retardation experiments to single- and double-stranded DNA, RNA and DNA-RNA heteroduplex under high-salt conditions¹⁰, that is, the range of nucleic acids found in an elongation bubble. Intact TFIIS stimulates nascent transcript cleavage and antitermination of RNAPII complexes arrested at various template sequences¹¹⁻¹⁴. In accord with this broad range of biological targets, the TFIIS domain binds random nucleic-acid sequences but has preferential affinity for oligopyrimidine single strands¹⁰. As template DNA sequences in RNAPII pause and termination sites are frequently A-rich^{2,5-8,10,30}, potential interactions between the Zn ribbon and U-rich single-stranded RNA and RNA-DNA hetero-oligomers will be of particular interest. The TFIIS RNAPII-binding domain may contribute additional specificity to the interaction between the intact protein and the stalled elongation complex¹⁰.

The structure of the TFIIS nucleic-acid binding domain thus defines a novel Zn-dependent architecture. Designated the Zn ribbon, this structure consists of a three-stranded β -sheet and disordered loop. Analogous Zn-binding sites are predicted in a variety of other proteins which interact with non-canonical nucleic-acid structures. The present study provides a foundation for analysis of the interaction between TFIIS and oligonucleotide models of an elongation bubble⁴. Because of the presumed role of TFIIS in viral and proto-oncogene expression, its structure may also provide a target for antiviral and antineoplastic drug design. □

Received 12 February; accepted 28 June 1993.

1. Yanofsky, C. J. *biol. Chem.* **263**, 609-612 (1988).
2. Rice, G. A., Kane, C. M. & Chamberlin, M. J. *Proc. natn. Acad. Sci. U.S.A.* **88**, 4245-4249 (1991).
3. von Hippel, P. H. & Yager, T. D. *Science* **255**, 809-812 (1992).
4. Daube, S. S. & von Hippel, P. H. *Science* **258**, 1320-1324 (1992).
5. Rougvie, A. E. & Lis, J. T. *Cell* **54**, 795-804 (1988).
6. Bentley, D. L. & Groudine, M. *Nature* **321**, 702-706 (1986).
7. Reines, D., Chamberlin, M. J. & Kane, C. M. *J. biol. Chem.* **264**, 10799-10809 (1989).
8. Feinberg, M. D., Baltimore, D. & Frankel, A. D. *Proc. natn. Acad. Sci. U.S.A.* **88**, 4045-4049 (1991).
9. Yoo, O.-J. et al. *Nucleic Acids Res.* **19**, 1073-1079 (1991).
10. Agarwal, K. et al. *Biochemistry* **30**, 7842-7851 (1991).
11. Renies, D. J. *biol. Chem.* **267**, 3795-3800 (1992).
12. Izban, M. G. & Luse, D. S. *Genes Dev.* **6**, 1342-1356 (1992).
13. Wang, D. & Hawley, D. K. *Proc. natn. Acad. Sci. U.S.A.* **90**, 843-847 (1993).
14. Reines, D. & Mote, J. Jr. *Proc. natn. Acad. Sci. U.S.A.* **90**, 1917-1921 (1993).
15. Kassavetis, G. A. & Geliuschek, E. P. *Science* **259**, 944-945 (1993).
16. Clore, G. M. & Gronenborn, A. M. *Prog. NMR Spectrosc.* **23**, 43-92 (1991).
17. Lee, M. S., Gippert, G. P., Soman, K. V., Case, D. A. & Wright, P. E. *Science* **245**, 635-637 (1989).
18. Hard, T. et al. *Science* **249**, 157-160 (1990).
19. Pavletich, N. P. & Pabo, C. O. *Science* **252**, 809-817 (1991).
20. Luisi, B. F. et al. *Nature* **352**, 497-505 (1991).
21. Marmorstein, R., Carey, M., Ptashne, M. & Harrison, S. C. *Nature* **356**, 408-414 (1992).
22. Ohkuma, Y. et al. *Nature* **354**, 398-401 (1991).
23. Dunn, J. J. & Studier, F. W. *J. molec. Biol.* **166**, 477-535 (1983).
24. Qian, X., Yoon, H.-S., Jeon, C.-J., Agarwal, A. & Weiss, M. A. *Biochemistry* (in the press).
25. Woychik, N.A., Lane, W. S. & Young, R. A. *J. biol. Chem.* **266**, 19053-19055 (1991).
26. Vallee, B. L., Coleman, J. E. & Auld, D. S. *Proc. natn. Acad. Sci. U.S.A.* **88**, 999-1003 (1991).
27. Somers, W. J. & Phillips, S. E. V. *Nature* **359**, 387-393 (1992).
28. Nikolov, D. B. et al. *Nature* **360**, 40-46 (1992).
29. Dombroski, A. J., Waiter, W. A., Record, M. T. Jr, Siegle, D. A. & Gross, C. A. *Cell* **70**, 501-512 (1992).
30. Qian, X. & Weiss, M. A. *Biochemistry* **31**, 7463-7476 (1992).

ACKNOWLEDGEMENTS. We thank M. Izban, D. Luse and D. Reines for communication of results before publication, S. N. Gozani for database search and discussion, J. P. Lee for assistance with NMR measurements, A. Brunker, T. Havel and M. Nilges for computer programs and advice, Molecular Simulations, Inc. (Waltham, Massachusetts) for the program *Quanta* used to generate Fig. 2d, and K. Struhl and C. Richardson for discussion. Coordinates and NMR derived restraint list have been deposited in the Brookhaven Data Bank. NMR spectra were obtained at Harvard Medical School. This work was supported in part by grants from the Council for Tobacco Research (M.A.W.) and NIH (K.A.). C.J.J. and H.-S.Y. contributed equally to this work.

CORRECTIONS

Segregation of global and local motion processing in primate middle temporal visual area

Richard T. Born & Roger B. H. Tootell

Nature **357**, 497-499 (1992)

IN experiments that used the 2-deoxyglucose technique to label the band/interband pattern in the middle temporal visual area of the owl monkey, animals were injected with 50 $\mu\text{g kg}^{-1}$ of methamphetamine ~10 min before infusion of 2-deoxyglucose. This was not included in the reference cited in our paper as a description of the method (ref. 22). We have subsequently repeated the 2-deoxyglucose labelling of the band/interband patterns without methamphetamine in two animals and have found no obvious differences in labelling in the two conditions. However, we wish to clarify the methods used in our letter. □

Continuous c-fos expression precedes programmed cell death *in vivo*

Richard J. Smeyne, Montserrat Vendrell, Michael Hayward, Suzanne J. Baker, Graham G. Miao, Karl Schilling, Linda M. Robertson, Tom Curran & James I. Morgan

Nature **363**, 166-169 (1993)

IN Fig. 1d of this letter, the structure marked EnK (enamel knot) was misidentified and should be the mesenchymal dental papilla. This does not alter the major conclusions of the paper. □

ERRATUM

Modulation of the cGMP-gated channel of rod photoreceptor cells by calmodulin

Yi-Te Hsu & Robert S. Molday

Nature **361**, 76-79 (1993)

IN Fig. 3 of this letter, the x-axis label should read $[\text{Ca}^{2+}]$ (nM) and not $[\text{Ca}^{2+}]$ (mM) as published. □

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